Gustav Steinhoff *Editor*

Regenerative Medicine

From Protocol to Patient

Second Edition

Regenerative Medicine

Gustav Steinhoff
Editor

Regenerative Medicine

From Protocol to Patient

Second Edition

Springer

Editor
Gustav Steinhoff
Department of Cardiac Surgery and
Reference and Translation Center
of Cardiac Stem Cell Therapy (RTC)
Medical Faculty
University of Rostock
Rostock, Germany

ISBN 978-94-007-5689-2 ISBN 978-94-007-5690-8 (eBook)
DOI 10.1007/978-94-007-5690-8
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2013934586

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Foreword

Regenerative Medicine: From Protocol to Patient

Second edition

Regenerative Medicine is a fast emerging interdisciplinary field of research and clinical therapies on the repair, replacement or regeneration of cells, tissues or organs in congenital or acquired disease. This new field of research and clinical development focussing on stem cell science and regenerative biology is just starting to be the most fascinating and controversial medical development at the dawn of the twenty-first century. It can be envisaged that this development will establish completely new molecular and cellular techniques for medical therapy. An early rush of scientific development was set up more than 100 years ago by the physiology of blood regeneration (Hall and Eubanks 1896) and successful vascular surgical techniques for organ transplantation (Carrel and Guthrie 1905). However, the clinical realization of allogenic blood transfusion lasted until the discovery of the blood group antigens (Landsteiner and Levine 1928) and successful routine allogenic organ and bone marrow transplantation even until the end of the last century.

Similar to the field of allogenic cell and organ transplantation, it seems that *Regenerative Medicine* again condenses mankind's visions, hopes, and fears regarding medicine: Hopes of eternal life and effective treatment of uncurable disease as well as fears of misuse of technology and uncontrolled modifications of life are polarizing the scientific field. The development and public acceptance of new ethical and regulatory guidelines is a necessary process to support the further clinical development. Nevertheless, the vision of a new medicine using the regenerative power of biology to treat disease and restructure the organism is setting the aim for scientific, technological and medical development. Viewing the great expectations to restructure and regenerate tissue, organs or organisms the current attempts of scientist and physicians are still in an early phase of development.

The field of *Regenerative Medicine* has developed rapidly over the last 20 years with the advent of molecular and cellular techniques. This textbook on "Regenerative

Medicine: From Protocol to Patient" is aiming to explain the scientific knowledge and emerging technology as well as the clinical application in different organ systems and diseases. The international leading experts from four continents describe the latest scientific and clinical knowledge of the field of *Regenerative Medicine*. The process of translating science of *laboratory protocols into therapies* is explained in sections on regulatory, ethical and industrial issues. Patient needs are advocated by the proposition initiatives on the scientific development of new therapies.

This textbook is organized into five major parts: (*I*) *Biology of Tissue Regeneration*, (*II*) *Stem Cell Science and Technology*, (*III*) *Tissue Engineering*, *Biomaterials and Nanotechnology*, (*IV*) *Regenerative Therapies*, and (*V*) *Regulation and Ethics*.

We start with an overview on the *History of Regenerative Medicine*. This is followed by the part of *Biology of Tissue Regeneration*, which focuses on extracellular matrix, asymmetric stem cell division, stem cell niche regulation and stem cells during embryonic neurogenic development. The part on *Stem Cell Science and Technology* provides an overview on classification of stem cells and describes techniques for their derivation and culture. Basic properties of the cells are illustrated, and some areas of applications for these cells are discussed with emphasis on their possible future use in *Regenerative Medicine*.

The part of *Tissue Engineering*, *Biomaterials and Nanotechnology* focuses the development of technologies, which enable an efficient transfer of therapeutic genes and drugs exclusively to target cells and potential bioactive materials for clinical use. Chimerism, multifunctionalized nanoparticles and nanostructured biomaterials are described with regard to the technological development of new clinical cell technology.

The part on *Regenerative Therapies* gives a survey on the clinical development in the different organ systems. Disease specific approaches of new therapies, application technology, clinical achievements and limitations are described. The part on *Regulation and Ethics* describes the current legislation for clinical translation as the ethical and juridical development in different countries.

The textbook is aiming to give the student, the researcher, the health care professional, the physician, and the patient a complete survey on the current scientific basis, therapeutical protocols, clinical translation and practised therapies in *Regenerative Medicine*. On behalf of the sincere commitment of the international experts we hope to increase your knowledge, understanding, interest and support by reading the book.

After the successful introduction in 2011, the textbook has been actualized for the second edition. The basic science and clinical part has been extended by five new chapters.

Rostock, August 2012

Gustav Steinhoff (Editor)
and Hoang Tu-Rapp (Assistant Editor)

Literature

Carrel A, Guthrie CC (1906) Successful transplantation of both kidneys from a dog into a bitch with removal of both normal kidneys from the latter. Science 23(584):394–395
Hall WS, Eubank MD (1896) The regeneration of the blood. J Exp Med 1(4):656–676
Landsteiner K, Levine P (1928) On individual differences in human blood. J Exp Med 47(5):757–775

Prof. Dr. med. Gustav Steinhoff
Professor of Cardiac Surgery, Director,
Department of Cardiac Surgery
and
Reference and Translation Center of
Cardiac Stem Cell Therapy (RTC)
Medical Faculty, University of Rostock,
Schillingallee 35, 18057 Rostock, Germany

Dr. Hoang Tu-Rapp
Department of Cardiac Surgery, Medical Faculty,
University of Rostock, Schillingallee 35,
18057 Rostock, Germany

Contents

Contributors

Yoko Arai Ph.D. Alessandra Pierani Group, Genetics and Development of the Cerebral Cortex, Institute Jacques-Monod CNRS UMR 7592, Université Paris Diderot, Paris Cedex 13, France

Kristina Arvidson, D.D.S., Ph.D. Department of Clinical Dentistry – Centre for Clinical Dental Research, University of Bergen, Bergen, Norway Solna, Stockholm, Sweden

Takayuki Asahara, M.D., Ph.D. Department of Regenerative Medicine Science, Tokai University School of Medicine, Isehara, Kanagawa, Japan

Group of Vascular Regeneration Research, Institute of Biomedical Research and Innovation (IBRI)/RIKEN Center for Developmental Biology, Chuo-ku, Kobe, Japan

Oliver Bachmann, M.D. Department of Gastroenterology, Hepatology and Endocrinology, Centre for Internal Medicine, Hannover Medical School (MHH), Hannover, Germany

Augustinus Bader, M.D. Stem cell biology and cell technology, Medical Faculty, Biotechnological-Biomedical Center (BBZ), Leipzig University, Leipzig, Germany

Silvia Baiguera, Ph.D. BIOAIR (Laboratory of Biomolecular and Bioengineering Airway), Department of General Thoracic and Regenerative Surgery and Intrathoracic Biotransplantation, University Hospital Careggi, Florence, Italy

Dorairajan Balasubramanian, Ph.D. Prof Brien Holden Eye Research Centre, University of Hyderabad, L V Prasad Eye Institute, Banjara Hills, Hyderabad, Andhra Pradesh, IndiaUniversity of Hyderabad, Banjara Hills, Hyderabad, Andhra Pradesh, India

Murali M.S. Balla Champalimaud Translational Centre for Eye Research, Hyderabad Eye Research Foundation, L.V. Prasad Eye Institute, Banjara Hills, Hyderabad, Andhra Pradesh, India

Sharmila A. Bapat, Ph.D., FNASc National Centre for Cell Science, Pune, Maharashtra, India

Gregor Becker, Ph.D. Group for Bio-Ethics in Life Sciences (GBLS), Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

Arne Berner, M.D. Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, QLD, Australia

Ute Bissels, Ph.D. Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Andreas Bosio, Ph.D. Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Christoph Brenner, M.D. Department of Internal Medicine I, University of Munich – Campus Grosshadern, Munich, Germany

Federico Calegari, Ph.D. Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

DFG-Center and Cluster of Excellence for Regenerative Therapies, Dresden University of Technology, Dresden, Germany

Tobias Cantz, M.D. Junior Research Group "Stem Cell Biology", Cluster of Excellence REBIRTH, Hannover Medical School, Hannover, Germany

Maurizio C. Capogrossi, M.D. Laboratorio di Patologia Vascolare, Istituto Dermopatico dell' Immacolata, (IDI)-Istituto di Ricovero e Cura a CarattereScientifico (IRCCS), Rome, Italy

Ilya Chuykin, Ph.D. Department of Cardiology and Pneumology, Georg-August-University Göttingen, Göttingen, Germany

Mary L. Clarke, Ph.D. Institute of Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, UK

Michele Cottler-Fox, M.D. Department of Clinical Dentistry – Centre for Clinical Dental Research, University of Bergen, Bergen, Norway

Robert David, Ph.D. Department of Internal Medicine I, University of Munich – Campus Grosshadern, Munich, Germany

Georg N. Duda, Ph.D. Julius Wolff Institute and Centre for Musculoskeletal Surgery, Berlin-Brandenburg Centre for Regenerative Therapy, Charité, Berlin, Germany

Dominik Eckardt, Ph.D. Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Mikael C.O. Englund, Ph.D. Cellectis Stem Cells, Dundee, UK

Joshua O. Eniwumide, Ph.D. Julius Wolff Institute and Centre for Musculoskeletal Surgery, Berlin-Brandenburg Centre for Regenerative Therapy, Charité, Berlin, Germany

Jonathan Frampton, B.A., Ph.D. Institute of Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, UK

Wolfgang-Michael Franz, M.D. Department of Internal Medicine I, University of Munich – Campus Grosshadern, Munich, Germany

Mathias Freund, M.D., Ph.D. 3rd Medical Department, Hematology, Oncology, Palliative Medicine, Center of Medicine, Rostock University Hospitals, Rostock, Germany

Changyou Gao, Ph.D. Department of Polymer Science and Engineering, Zhejiang University, Hangzhou, Zhejiang Province, People's Republic of China

Anna Grabinski Paris, France

May Griffith, Ph.D., MBA Integrative Regenerative Medicine (IGEN) Centre, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

Kaomei Guan, Ph.D. Department of Cardiology and Pneumology, Georg-August-University Göttingen, Göttingen, Germany

Christina Irene Günter, M.D. Clinical Trials Research Group, Clinic for Plastic and Hand Surgery, University Hospital Rechts der Isar, Technische Universität München, Munich, Germany

Hermann Haller, M.D. Department of Nephrology and Hypertension, Hannover Medical School, Hannover, Germany

Sølve Hellem, D.D.S., Ph.D. Department of Clinical Dentistry – Centre for Clinical Dental Research, University of Bergen, Bergen, Norway

Ottmar Herchenröder, Ph.D. Department of Vectorology and Experimental Gene Therapy, Biomedical Research Center, University of Rostock Medical School, Rostock, Germany

Frank Hirth, B.Sc., M.Sc., Ph.D., PD Department of Neuroscience, MRC Centre for Neurodegeneration Research, Institute of Psychiatry, King's College London, London, UK

Anthony D. Ho, M.D., FRCPC Department of Internal Medicine V, Heidelberg University, Heidelberg, Germany

Raymund E. Horch, M.D. Department of Plastic and Hand Surgery, University of Erlangen, Erlangen, Germany

Dietmar W. Hutmacher, Ph.D., M.B.A., M.Sc. Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, QLD, Australia

Wieland B. Huttner, M.D. Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany

Johan Hyllner, Ph.D. Cellartis AB, Göteborg, Sweden

Razvan Iacob, M.D. University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania

Masaaki Ii, M.D., Ph.D. Department of Pharmacology, Group of Translational Stem Cell Research, Osaka Medical College, Takatsuki, Osaka, Japan

Lavinia Iuliana Ionescu, M.D., Ph.D. Department of Physiology, 3032 Katz Group Centre for Pharmacy and Health Research, University of Alberta, Edmonton, AB, Canada

Tohru Itoh, Ph.D. Laboratory of Cell Growth and Differentiation, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan

Elmar Jäckel, M.D. Department of Gastroenterology, Hepatology and Endocrinology, Centre for Internal Medicine, Hannover Medical School (MHH), Hannover, Germany

Kewal K. Jain, M.D., FRACS, FFPM Jain PharmaBiotech, Basel, Switzerland

Nopporn Jongkamonwiwat, Ph.D. Centre for Stem Cell Biology and Department of Biomedical Sciences, University of Sheffield, Sheffield, UK

Emilia Kaivosoja, M.Sc. Department of Medicine, Institute of Clinical Medicine, Helsinki, Finland

Atsuhiko Kawamoto, M.D., Ph.D. Unit of Regenerative Medicine, Institute of Biomedical Research and Innovation/RIKEN Center for Developmental Biology, Chuo-ku, Kobe, Japan

Seung U. Kim, M.D., Ph.D. Division of Neurology, Department of Medicine, UBC Hospital, University of British Columbia, Vancouver, BC, Canada

Medical Research Institute, Chung-Ang University College of Medicine, Seoul, South Korea

Michelle Klose (Stange), M.D. Department of Gastroenterology, Hepatology and Endocrinology, Centre for Internal Medicine, Hannover Medical School (MHH), Hannover, Germany

Yrjö T. Konttinen, M.D., Ph.D. Department of Medicine, Institute of Clinical Medicine, Helsinki, Finland

Anjali P. Kusumbe National Centre for Cell Science, Pune, Maharashtra, India

Andreas Lendlein, Prof. Center for Biomaterial Development, Helmholtz-Zentrum Geesthacht, Teltow, Germany

Julius-Wolf Institute and Berlin-Brandenburg Centre for Regenerative Therapies (BCRT), Charité, Berlin, Germany

Jaakko Levón, B.Sc. Department of Medicine, Institute of Clinical Medicine, Helsinki, Finland

Wenzhong Li, Dr.-ing (NUS) Center for Biomaterial Development and Berlin Brandenburg Center for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Teltow, Germany

Paolo Macchiarini, M.D., Ph.D. Department of General Thoracic and Regenerative Surgery, and Intrathoracic Biotransplantation, University Hospital Careggi, Florence, Italy

Advanced Center for Translational Regenerative Medicine (ACTREM), ENT (CLINTEC), Karolinska Institutet, Huddinge, Stockholm, Sweden

University College London, London, UK

Hans-Günther Machens, M.D. Clinic for Plastic and Hand Surgery, University Hospital Rechts der Isar, Technische Universitaet Muenchen, Munich, Germany

Zygmunt Mackiewicz, Ph.D., D.Sc. Department of Histology and Embryology, Medical University of Bialystok, Bialystok, Poland

Michael P. Manns, M.D. Department of Gastroenterology, Hepatology and Endocrinology, Centre for Internal Medicine, Hannover Medical School (MHH), Hannover, Germany

Indumathi Mariappan, Ph.D. Sudhakar & Sreekanth Ravi Stem Cell Biology Laboratory, Prof Brien Holden Eye Research Centre, L.V. Prasad Eye Institute, Banjara Hills, Hyderabad, Andhra Pradesh, India

Almudena Martinez-Fernandez, PharmD, Ph.D. Marriott Heart Disease Research Program, Division of Cardiovascular Diseases, Departments of Medicine, Molecular Pharmacology and Experimental Therapeutics, and Medical Genetics, Mayo Clinic, Rochester, MN, USA

Christopher R. McLaughlin, Ph.D. Michael G. DeGroote School of Medicine, McMaster University, Hamilton, ON, Canada

Thomas Mittlmeier, M.D. Department of Trauma and Reconstructive Surgery, University of Rostock, Rostock, Germany

Atsushi Miyajima, Ph.D. Laboratory of Cell Growth and Differentiation, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan

Nonsikelelo Mpofu-Mätzig, Ph.D. Department of Gastroenterology, Hepatology and Endocrinology, Centre for Internal Medicine, Hannover Medical School (MHH), Hannover, Germany

Kamal Mustafa, D.D.S., Ph.D. Department of Clinical Dentistry – Centre for Clinical Dental Research, University of Bergen, Bergen, Norway

Nageshwar D. Reddy, M.D., D.M., D.Sc, FAMS FRCP Asian Institute of Gastroenterology, Hyderabad, India

Timothy J. Nelson, M.D., Ph.D. Marriott Heart Disease Research Program, Division of Cardiovascular Diseases, Departments of Medicine, Molecular

Pharmacology and Experimental Therapeutics, and Medical Genetics, Mayo Clinic, Rochester, MN, USA

Keisuke Okita, Ph.D. Center for iPS Cell Research and Application(CiRA), Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto, Japan

Fumitaka Osakada, Ph.D. Systems Neurobiology Laboratory, The Salk Institute for Biological Studies, La Jolla, San Diego, CA, USA

Michael Ott, M.D. Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany

TWINCORE, Centre for Experimental and Clinical Infection Research, Hannover, Germany

Maurizio Pesce, Ph.D. Laboratorio di Ingegneria Tissutale Cardiovascolare, Centro Cardiologico Monzino, IRCCS, Milan, Italy

Naresh Polisetti, Ph.D. Department of Ophthalmology, Electron Microscopy and Cell Biology, University of Erlangen-Nuernberg, Erlangen, Germany

Gianluca Polvani, Ph.D. Dipartimento di Chirurgia Cardiovascolare Laboratorio di Biologia, Vascolare e Medicina Rigenerativa, Centro Cardiologico Monzino-IRCCS, Università degli Studi di Milano, Milan, Italy

Elias Polykandriotis, M.D. Department of Plastic and Hand Surgery, University of Erlangen, Erlangen, Germany

Giulio Pompilio, M.D., Ph.D. Dipartimento di Chirurgia Cardiovascolare Laboratorio di Biologia, Vascolare e Medicina Rigenerativa, Centro Cardiologico Monzino-IRCCS, Università degli Studi di Milano, Milan, Italy

Laurentiu M. Popescu, M.D., Ph.D. "Victor Babes" National Institute of Pathology, Buchrest, Romania

Department of Cellular and Molecular Medicine, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania

Brigitte M. Pützer, M.D., Ph.D. Department of Vectorology and Experimental Gene Therapy, Biomedical Research Center, University of Rostock Medical School, Rostock, Germany

G.V. Rao, M.S., MAMS, FRCS Asian Institute of Gastroenterology, Hyderabad, India

Julia Reetz (Bäder) Department of Vectorology and Experimental Gene Therapy, Biomedical Research Center, University of Rostock Medical School, Rostock, Germany

Johannes C. Reichert, M.D., Ph.D. Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, QLD, Australia

Department of Trauma, Hand, Plastic and Reconstructive Surgery, Julius-Maximilians-University, Würzburg, Germany

Marcelo N. Rivolta, M.D., Ph.D. Centre for Stem Cell Biology and Department of Biomedical Sciences, University of Sheffield, Sheffield, UK

Joachim Rychly Laboratory of Cell Biology, University of Rostock, Rostock, Germany

Siamak Saifzadeh, DVM., Ph.D. Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, QLD, Australia

Virender S. Sangwan, M.D. Cornea and Anterior Segment, Ocular Immunology & Uveitis Services, Prof Brien Holden Eye Research Centre, L.V. Prasad Eye Institute, Hyderabad, Andhra Pradesh, India

Peter Sartipy, Ph.D. Cellartis AB, Göteborg, Sweden

M. Sasikala, Ph.D., MAMS Institute of Basic Sciences and Translational Research, Asian Healthcare Foundation, Hyderabad, India

Anke Schmidt, Ph.D. Zentrum für Innovationskompetenz (ZIK) plasmatis, INP Greifswald e.V. – VON DER IDEE BIS ZUM PROTOTYP, Greifswald, Germany

Michael Schmitt, M.D., Ph.D., MHBA Cellular Immunotherapy, University Clinic Heidelberg, Heidelberg, Germany

Michael Schroeter, Ph.D. Centre for Biomaterial Development, Institute of Polymer Research, Helmholtz-Zentrum Geesthacht, Teltow, Germany

Sebastian C. Sethe, Ph.D. Lawford Davies Denoon, London, UK

Amar Deep Sharma, Ph.D. Junior Research Group "Stem Cell Biology", Cluster of Excellence REBIRTH, Hannover Medical School, Hannover, Germany

Michael Sittinger, Ph.D. Department of Rheumatology and Clinical Immunology, Tissue Engineering Laboratory and Berlin-Brandenburg Center for Regenerative Therapies, Charité-Universitätsmedizin Berlin, Berlin, Germany

Sajoscha A. Sorrentino, M.D. Department of Radiology, Hannover Medical School, Hannover, Germany

Michael Stauske, M.Sc. Department of Cardiology and Pneumology, Georg-August-University Göttingen, Göttingen, Germany

Vasily Stegaev, M.D. Department of Medicine, Institute of Clinical Medicine, Helsinki, Finland

Gustav Steinhoff, M.D., Ph.D. Department of Cardiac Surgery and Reference and Translation Center of Cardiac Stem Cell Therapy (RTC), Medical Faculty, University of Rostock, Rostock, Germany

Ioannis Stratos, M.D. Department of Trauma and Reconstructive Surgery, University of Rostock, Rostock, Germany

Bodo Eckehard Strauer, M.D., FESC, FACC Department of Cardiology, Pneumology and Angiology, Heinrich-Heine-University of Düsseldorf, Düsseldorf, Germany

Pei Sun, Ph.D. Zhongguancun Life Science Park, National Institute of Biological Sciences, Beijing, China

Kazutoshi Takahashi, Ph.D. Center for iPS cell Research and Application (CiRA), Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto, Japan

Masayo Takahashi, M.D., Ph.D. Laboratory for Retinal Regeneration, Center for Developmental Biology, RIKEN, Chuo-ku, Kobe, Japan

Hinako Takase, Ph.D. Department of Developmental Biology, Howard Hughes Medical Institute, School of Medicine, Stanford University, Stanford, CA, USA

Minoru Tanaka, Ph.D. Laboratory of Cell Growth and Differentiation, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan

Manu Tandan, M.D., D.M. Asian Institute of Gastroenterology, Hyderabad, India

Andre Terzic, M.D., Ph.D. Marriott Heart Disease Research Program, Division of Cardiovascular Diseases, Departments of Medicine, Molecular Pharmacology and Experimental Therapeutics, and Medical Genetics, Mayo Clinic, Rochester, MN, USA

Bernard Thébaud, M.D., Ph.D. Department of Pediatrics, Northern Alberta Neonatal Program, Women and Children's Health Research Institute, Cardio-Vascular Research Center, Pulmonary Research Group, University of Alberta, Edmonton, AB, Canada

Veli-Matti Tiainen, Ph.D. ORTON Orthopaedic Hospital, ORTON Foundation, Helsinki, Finland

Gudrun Tiedemann, Ph.D. Reference and Translation Center for Cardiac Stem Cell Therapy (RTC), University of Rostock, Rostock, Germany

Shubha Tiwari, M.Pharm. Sudhakar and Sreekant Ravi Stem Cell Biology Laboratory, Prof Brien Holden Research Centre, L.V. Prasad Eye Institute, Hyderabad, India

Gerben M. van Buul, M.D. Departments of Orthopaedics and Radiology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Gerjo J.V.M. van Osch, Ph.D. Departments of Orthopaedics and Radiology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Geeta K. Vemuganti, M.D., DNB, FAMS, FICP School of Medical Sciences, University of Hyderabad, Gachibowli, Hyderabad, Andhra Pradesh, India

Hanoch Daniel Wagner, Ph.D. Department of Materials and Interfaces, Weizmann Institute of Science, Rehovot, Israel

Wolfgang Wagner, M.D., Ph.D. Institute for Biomedical Engineering, University of Aachen Medical School, Aachen, Germany

Chenhui Wang Zhongguancun Life Science Park, National Institute of Biological Sciences, Beijing, China

Pei Wen Zhongguancun Life Science Park, National Institute of Biological Sciences, Beijing, China

Britt Wildemann, Prof. Julius-Wolf Institute and Berlin-Brandenburg Centre for Regenerative Therapies (BCRT), Charité, Berlin, Germany

Patrick Wuchter, M.D. Department of Internal Medicine V, Heidelberg University, Heidelberg, Germany

Rongwen Xi, Ph.D. Zhongguancun Life Science Park, National Institute of Biological Sciences, Beijing, China

Satsuki Yamada, M.D., Ph.D. Marriott Heart Disease Research Program, Division of Cardiovascular Diseases, Departments of Medicine, Molecular Pharmacology and Experimental Therapeutics, and Medical Genetics, Mayo Clinic, Rochester, MN, USA

Shinya Yamanaka, M.D., Ph.D. Center for iPS cell Research and Application (CiRA), Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto, Japan

Department of Stem Cell Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

Yamanaka iPS Cell Special Project, Japan Science and Technology Agency, Kawaguchi, Japan

Gladstone Institute of Cardiovascular Disease, San Francisco, USA

Maria Patapia Zafiriou, Ph.D. Department of Pharmacology, Heart Research Center Goettingen, University Medical Center Goettingen, Goettingen, Germany

Laura C. Zelarayán, Ph.D. Department of Pharmacology, Heart Research Center Goettingen, University Medical Center Goettingen, Goettingen, Germany

Jie Zhou, Ph.D. R&D Center, Jushi Group Co., Ltd., Tongxiang City, Zhejiang Province, People's Republic of China

Wolfram-Hubertus Zimmermann, M.D. Department of Pharmacology, Heart Research Center Goettingen, University Medical Center Goettingen, Goettingen, Germany

Chapter 1
History of Regenerative Medicine

Raymund E. Horch, Laurentiu M. Popescu, and Elias Polykandriotis

Abstract Generation and regeneration as an answer to disease are far from being a new idea. Philosophers, naturalists and scientists were intrigued by the marvels of regeneration seen in nature. By the middle of the 1990s life scientists thought we were only a few years away from bioartificial organs grown in a Petri dish. However, by the dawn of the new millennium it became clear that the mechanistic approach dictated by tissue engineering so far, had neglected issues of vascularization. Processes of angiogenesis were central to homeostasis, bioassimilation and biointegration of tissue engineered constructs. Furthermore, the field of tissue engineering had evolved into something vast, encompassing satellite technologies that were becoming separate science sectors. Advances in genetical engineering, stem cell biology, cloning, biomaterials and biomedical devices to name a few, would come to play a major role of their own – tissue engineering had become a part of a bigger whole. Regenerative medicine is the collective field to shelter these technologies "*…that seeks to develop functional cell, tissue, and organ substitutes to repair, replace or enhance biological function that has been lost due to congenital abnormalities, injury, disease, or aging*"

R.E. Horch, M.D. (✉) • E. Polykandriotis, M.D.
Department of Plastic and Hand Surgery, University of Erlangen,
Krankenhausstr. 12, 91054 Erlangen, Germany
e-mail: raymund.horch@uk-erlangen.de

L.M. Popescu, M.D., Ph.D.
"Victor Babes" National Institute of Pathology,
99-101 Splaiul Independentei, 050096 Buchrest, Romania

Department of Cellular and Molecular Medicine,
"Carol Davila" University of Medicine and Pharmacy,
P.O. Box 35-29, Bucharest 35, Romania

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_1,

1.1 Introduction

"Those who cannot learn from history are doomed to repeat it", claimed the philosopher G. Santayana in his book "The life of reason" (Santayana 1905). Although this statement reminds somehow of a cliché and its essence is being constantly disputed through the ages, one could hardly find a better example to report upon, other than the case of regenerative medicine. It is widely admitted that the very term of "Regenerative medicine" was coined to express a need for reorientation (Table 1.1). By the end of the twentieth century, biotechnology firms had maneuvered themselves into a dead-end financially, as well as conceptually (Mason 2007). Furthermore, the field of tissue engineering had evolved into something vast, encompassing satellite technologies that were becoming separate science sectors. Advances in genetical engineering, stem cell biology, cloning, biomaterials and biomedical devices to name a few, would come to play a major role of their own – tissue engineering had become a part of a bigger whole. And it is undisputable that biologicals will be the future (Mason and Dunnill 2008). To quote Paul Kemp: "hype, hubris and hyperbole aside – regenerative medicine will make a real and positive difference…" (Kemp 2006). But where did it all start?

1.2 Regenerative Medicine in the Ancient World

In his *Theogony*, Hesiod (eighth century BC) introduces Prometheus (Fig. 1.1) having created man out of clay and providing him with fire as a source of knowledge. "Hear the sum of the whole matter in the compass of one brief word – every art possessed by man comes from Prometheus."(Aeschylus 1832). By doing that, Prometheus had provoked the wrath of Zeus. He had Prometheus carried to Mount Caucasus (or the Carpathian mountains) where an eagle (often mistaken as a vulture) by the name of Ethon would pick at his liver; it would grow back each day and the eagle would eat it again. His torture lasted 30,000 years until he was freed by Hercules (Fig. 1.1). Interestingly enough, the liver is generally speaking the only of the human organs to regenerate itself spontaneously in the case of lesion.[1] The ancient Greeks were well aware of this, hence they named liver (Greek: hēpar, ήπαρ) after hēpaomai (ηπάομαι), meaning to "repair oneself".

Later on, Aristotle devised two scripts dealing with generation and regeneration in the animal realm. In his "Generation of animals" he related early development with regenerative potential, whereas in "The history of animals" he made observations on regeneration on the limb of salamanders and deer antlers (Aristotle 1984). He propagated that biological form originates from undifferentiated matter and clearly favoured what would later be described as "epigenesis" (Fig. 1.2).

[1] Now the phenomenon of desquamation of the intestinal epithelium and the epidermis has been described. The intestinal epithelium is completely regenerated in 4–5 days. The total regeneration of the epidermis takes 4 weeks. This may mean that for a life expectancy of 77 years, the human epidermis is regenerated 1,000 times.

Table 1.1 Scientometric data based on Thomson Reuters released information (August 2009)

	Tissue engineering	Regenerative medicine
Total No of PubMed Papers (starting in.)	14,517 (since 1988)	2,197 (since 2001)
Year of maximum	–	2008 (ca 600)
Most cited paper	2,452 citations	1,366 citations
Top 100 papers	At least 200 citations each	At least 40 citations each

Fig. 1.1 Prometheus. "Prometheus", Gustave Moreau 1868 (Musée Gustave Moreau, Paris). According to some investigators, his torture held for 30,000 years. After having provoked the wrath of Zeus, the eagle Ethon, picked at his liver every night. During the day the liver would regenerate

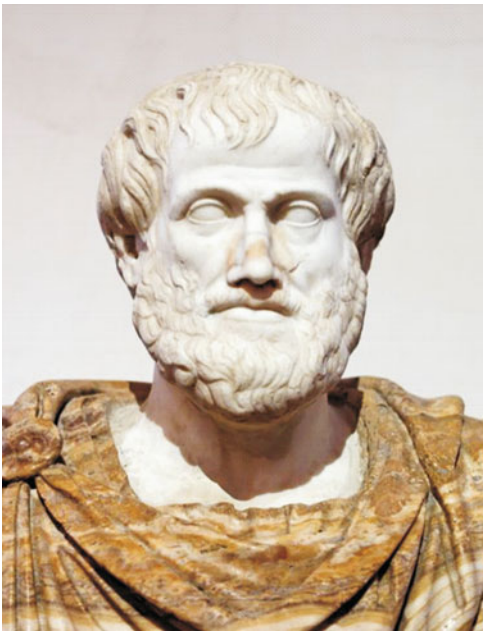


Fig. 1.2 Aristotle. Aristotle's bust. Roman copy from the bronze original by Lyssipos (fifth century B.C.). (Ludovisi Collection). Aristotle wrote two major works on generation and regeneration in the animal realm. He related early development with regenerative potential and propagated that biological form originates from undifferentiated matter (epigenesis)

In the biblical tradition "the Lord God then built up into a woman the rib that he had taken from the man" (Jones 1988). The quest for tissue replacement was even more graphically demonstrated in the tradition of Cosmas and Damian. Their practice of medicine and surgery in Asia Minor without fee (hence called 'Anagyroi,' without silver) and their martyrdom in Aegea, in Cilicia made a lasting impression upon the early Church. The grafting by these physician-surgeons of a moor's leg in replacement of a patient's diseased leg, and his surprise at finding himself possessed of two sound legs, his own white, and the other black, has been the subject of numerous paintings the majority of which depict the brothers in long robes, holding surgical instruments, boxes of salves, gallipots, or other medical appliances (Matthews 1968) (Fig. 1.3). Graveyards from the Paracas and Parachamac regions in Peru provide ample evidence that pre-Incan surgeons were performing trephination in great numbers as early as 3000 BC. A survey of more than 10,000 mummies from prehistoric Peru demonstrated that roughly 6% showed cranial trephination. There is strong evidence that the occasional cranioplasty was also performed. Trephined Incan skulls have been discovered adjacent to shells, gourds, and silver or gold plates (Asenjo 1963).

1.3 Regeneration in Early Research

Until the middle of the eighteenth century the motive power of biological organisms was thought to be an abstract vital force. Descartes (1596–1650) in his L' Homme postulated that the body works like a machine and biological phenomena are void of

Fig. 1.3 Saints Cosmas and Damian. "Transplantation of a leg by Saints Cosmas and Damian, assisted by angels", early sixteenth century (Stuttgart, Germany). According to the tradition of Cosmas and Damian these saints grafted a moors leg as a replacement of a patient's diseased leg

a divine meaning but can be explained by means of their physical properties. Lavoisier (1743–1794) postulated further on, that function and viability of organisms depended on chemical processes that could be reproduced in the laboratory. During the same time phenomena of generation and regeneration intrigued scientists and divided them into two distinct camps. Preformationists supported that appendages to be regenerated and organisms to be born pre-existed as miniatures at the site of interest. So, at the base of a severed lizard tail, in their conception a miniature tail was preformed and waited to be "activated" by an amputation. Likewise, in the sperm or in the ovum of the human there existed a miniature "homunculus" that grew into a newborn infant. This theory prevailed until the middle of the eighteenth century being concordant with the mechanistic framework provided at the time and did not come into a direct conflict with the Christian beliefs about divine involvement in the processes of life. On the contrasting end, came the Aristotelian thesis that undifferentiated matter was able to give rise to life. This theory had been

actually named “epigenesis” by William Harvey (1578–1657) in his work “on the generation of animals” grossly repeating on Aristotle’s works.

In the eighteenth century the process of regeneration in amphibians was matter of intense study. Abraham Trembley (1710–1784) produced several publications on the regenerative phenomena on freshwater polyps. He managed to obtain a clone of 50 polyps from one organism that he had quartered. He performed sections at every conceivable plane, contradicting preformational beliefs of the time (Dinsmore 1991). The question was posed: If the animal soul was the organizing and unifying element of life, how could a newly regenerated form arise? Reaumer and Spallanzani reported about their studies on crustaceans and salamanders respectively (Dinsmore 1991). The latter, being a great methodologist, expanded his research on a number of different organisms including frogs, toads, slugs and snails. He published his findings in 1768 in his work “Prodromo”. It was noted by Newth. “In 1768 the snails of France suffered an unprecedented assault. They were decapitated in their thousands by naturalists and others to find out whether or not it was true, as the Italian Spallanzani had recently claimed, that they would then equip themselves with new heads” (Newth 1958; Weaver and Garry 2008).

Until the end of the eighteenth century philosophical and religious debate linked to the science of regeneration was set aside, and epigenesis gained acceptance with the eventual ascendancy of epigenetic embryology.

The last years of the eighteenth century marked a new field of interest for regenerative medicine: organ transplantation. John Hunter (1728–1793) performed allograft transplantations between chickens as well as dental transplantation utilizing xenografts of human teeth to avian hosts. John Hunter was the most prominent surgeon and anatomist of his time. According to his instructions, his corpse was used for an anatomical dissection by his medical students on the day after his death.

At the beginning of the nineteenth century – following the 1794 description by B.L. in The Gentlemen’s Magazine in London of a forehead tissue transposition to restore the nose of a bullock cart driver named Cowasjee, that had been cut off as a punishment – the English surgeon Carpue was the first surgeon to apply methods of nasal reconstructions known to Indian surgeons for centuries (Carpue 1981). Dieffenbach described methods for reconstructions of several components of the face, as well as the anus and the urethra (Goldwyn 1968). Reverdin devised a method for transplantation of skin islets, similar to the later techniques for keratinocyte transplantation (Horch et al. 2001). Transplantational biology was investigated by experimental approaches: In 1824, Franz Riesinger attempted corneal transplants from rabbits to humans, which were not successful (Moffatt et al. 2005). In 1837 Samuel Bigger performed a corneal transplant from a lab gazelle to another Gazelle with full recovery. Later on, Schleiden and Schwann in 1838–1839 postulated the cell theory that was afterwards confirmed by Rudolf LK Virchow through microscopic observations. He stated in 1858 the famous “*omnis cellula ex cellula*”. Ultimately, the idea of cells being the elementary units of life being able to replicate themselves by division was born (Coleman 1978; Stocum 2006).

The eminent German pathologist Julius Cohnheim postulated in 1867 what became known as the “Cohnheim hypothesis”. He suggested that all of reparative

cells taking part in the regeneration of wounds come from the bloodstream (and therefore from the bone marrow) (Wohlrab and Henoch 1988).

At the end of the nineteenth century, Barth observed that upon autologous bone transplantation in hounds the vast majority of cells die and leave a scaffolding behind to be slowly repopulated by new host cells and an adequate neovascular network (Barth 1893).

Another very important advance was new knowledge on descriptive embryology that elicited a revolution in developmental biology. Even Darwin considered embryology as key to providing a special insight into evolution of forms, as seen in his correspondence to his friend Asa Grey.

If the living cell is the key to the tissue engineering of implantable parts and devices, then the advent of mammalian cell culture technology, i.e. the growing of mammalian cells out of the body, represents an event which ultimately opened the door for this field. Modern cell culture dates back to the early part of this century when a French scientist, Alexis Carrel, working at the Rockefeller Research Institute in New York, started a culture from a small slice of heart muscle taken from a chick embryo (Leff 1983). This culture continued for several decades, although along the way the heart muscle cells died out and only fibroblast cells continued to proliferate. Carrel's historic chick-cell culture finally was allowed to expire 34 years after it was started – and 2 years after his own death. Anecdotally and in the retrospect, his "immortal" adult cells might have benefited from interventions from Carrel's staff to keep the cells going and their teacher happy (Witkowski 1980). Now it is known, that according to the "Hayflick limit", cells in culture are not able to replicate more than 40–60 times and they are bound to display signs of senescence with successive passages (Hayflick 2007).

Cell culture has led to research which has paved the way for a number of important breakthroughs in the life sciences (Figs. 1.4 and 1.5). This includes the study of cellular processes, molecular biology and the ability to genetically manipulate cells, and the resulting development of new drugs, with much of recent drug-related research and product development being based on recombinant DNA technology (Nerem 1992). In the early 1970s, Dr. W.T. Green, a pediatric orthopaedic surgeon at Children's Hospital Boston, undertook a number of experiments to generate new cartilage using chondrocytes seeded onto spicules of bone and implanted in nude mice. Although unsuccessful, he correctly concluded that with the advent of innovative biocompatible materials it would be possible to generate new tissue by seeding viable cells onto appropriately configured scaffolds (Figs. 1.6 and 1.7). Several years later, Drs. John Burke, Massachusetts General Hospital, and Ioannis Yannas, MIT, collaborated in studies in both the laboratory and in humans to generate a tissue-engineered skin substitute using a collagen matrix to support the growth of dermal fibroblasts. Dr. Howard Green later transferred sheets of keratinocytes onto burn patients. Dr. Eugene Bell seeded collagen gels with fibroblasts, referring to them as contracted collagen gels. All of these examples represent seeds of the new discipline now known as tissue engineering (Vacanti 2006).

Modern research on embryonic stem cells originates from studies on teratocarcinomas arising from the gonads of imbred mice. These neoplasias displayed a

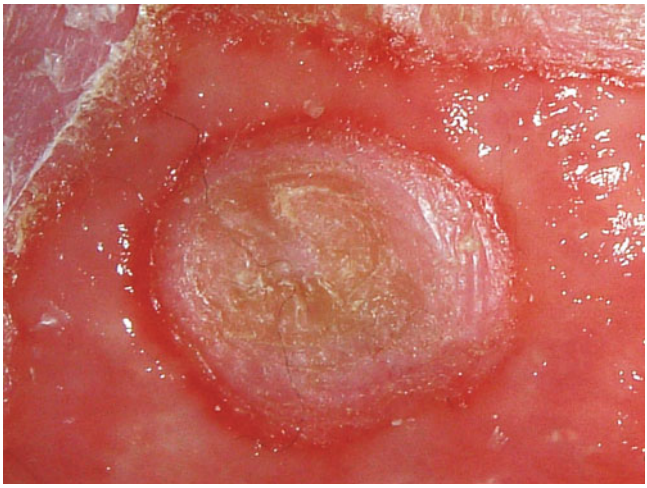


Fig. 1.4 Back from bench to bedside. Back from bench to bedside: small particles of skin inoculated into "biological" environment of wound showing expanding skin islet within a large wound 3 weeks after seeding

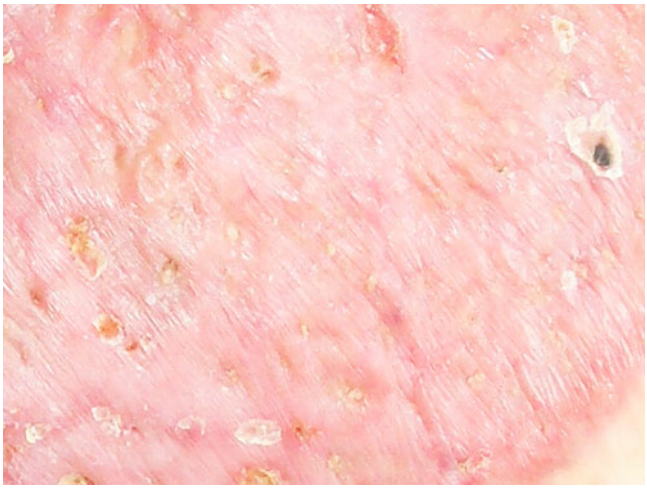


Fig. 1.5 Reepithelialisation. Eight weeks after skin particle seeding. Complete reepithelialisation is accomplished after "in situ culture" utilizing regenerative potentials of the human body

characteristic mixture of different tissues lined up next to each other randomly. By the end of the 1960s it was established that they originated from germ cells that were able to give rise to a plethora of different tissues. So the concept of pluripotency of germinal cells was introduced (Kleinsmith and Pierce 1964). From its potential to generate a multitude of different cells, the tumour cell was named

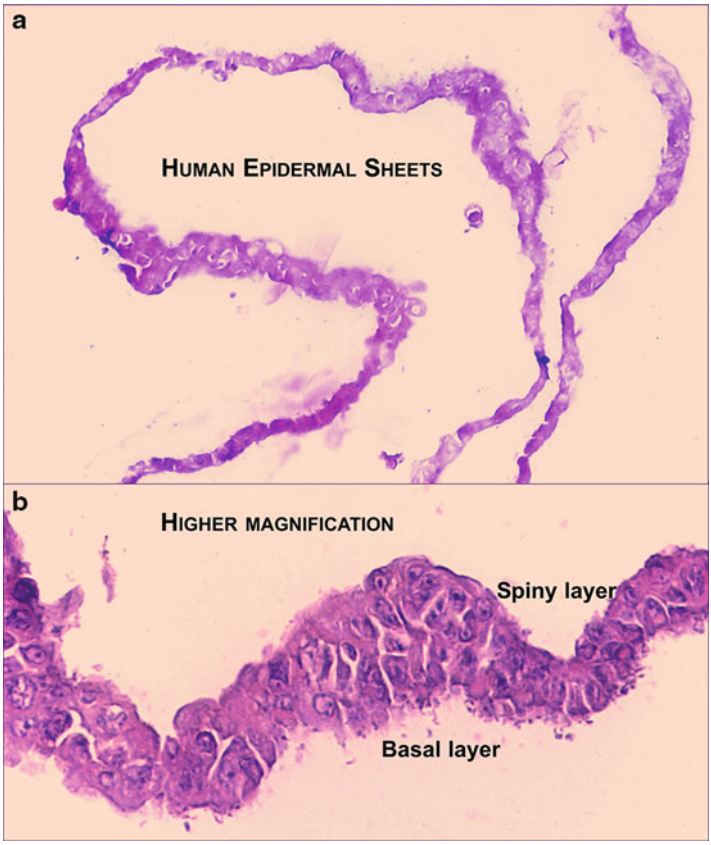


Fig. 1.6 Human epidermal sheets. Human epidermal sheets of autologous keratinocytes, obtained by cell culture (14 days). Light microscopy. (**a**) Objective 20×. (**b**) Objective 60× (Laboratory of Cellular and Molecular Medicine, Prof. L.M. Popescu, Bucharest)

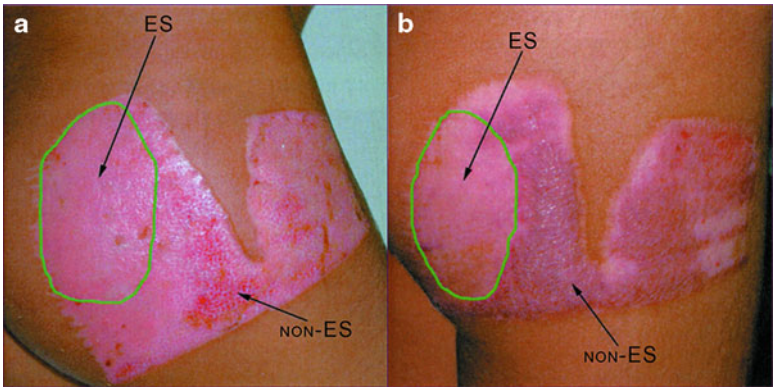


Fig. 1.7 Epidermal sheet treatment. Comparison between the regenerative processes of a donor area with (ES) or without (non-ES) epidermal sheet treatment. (**a**) Ten days after the application of the epidermal sheet. (**b**) Five weeks later (Courtesy of Prof. D. Enescu, Department of Plastic Surgery, Children's Hospital, Bucharest)

embryonal carcinoma stem cell (EC). Research with EC stem cells expanded considerably in the 1970s. In a series of experiments, chimeric mice were produced by injecting EC s into early blastocysts (Papaioannou et al. 1975). Interestingly enough, in most of the cases, the tumour cells succumbed to the environment around the developing embryo and they contributed to a perfectly normal mouse pup. Hence it was shown that their genetic code could be "reprogrammed" according to the influence of the environment. Furthermore, the EC stem cells in culture could be constantly kept undifferentiated by frequent splitting or left to differentiate when the culture became too dense and they piled up. However, the EC stem cells were inherently flawed displaying chromosome abnormalities and were unable to differentiate into sperm and egg cells. Since ectopic blastocyst injections were also found to generate teratomas it became soon evident that pluripotent cells could also be derived from blastocysts directly (Damjanov 1993). Soon the next logical step was undertaken, when Gail Martin (Martin 1981) in USA and Martin Evans (Evans and Kaufman 1981) in England generated in 1981 a stable diploid cell line that could generate every tissue of the adult body, including germ cells. Gail Martin referred to her cells as "embryonic stem cells" and gave them the nickname "ES cells".

The same line of advances had to be repeated for human cells: Human EC stem cell lines could be isolated and cultured from a rare tumor of the male testes, after orchiectomy procedures (Andrews 1988). However, these cells are always aneuploid and usually lack the capacity to differentiate into somatic tissue (Pera et al. 1989). Human ES cells were not available at this time. What was available, were blastocyst-derived embryonic cells from primates including rhesus monkeys and marmosets (Thomson et al. 1996). These cells displayed all favourable characteristics: they were diploid and were able to give rise to all three types of germinal layers, including germ cells. Their phenotype resembled that of the human EC and were distinctly different from the mice ES. All major technological advances for cultivation and characterization of human ES was achieved by the late 1990s – but their harvest was not yet possible.

In 1998 a major step was accomplished toward this direction marking the dawn of a new era. Couples undergoing treatment for extracorporeal fertilization donated a surplus of blastocysts for experimental purposes. James Thomson isolated and cultivated a human ES line from these blastocysts (Thomson et al. 1998).

Adult stem cells were also to enter the arena of biomedical research. The idea that bone marrow contained some kind of osteogenic precursor cells started in 1963 when Alexander Friedenstein (Petrakova et al. 1963) showed that by implanting pieces of bone marrow under the renal capsule, it was possible to obtain an osseous tissue. After this he and his co-workers revealed a series of in vivo studies in which the possible existence of stem cells in the bone marrow was shown. Almost 20 years later, Caplan gave these cells the name they have today, Mesenchymal Stem Cells. In 1994, the same author described that these cells, when placed in the adequate culture conditions, could be differentiated into cells with mesenchymal origin and eventually give origin to bone, cartilage, fat, muscle skin, tendon and other tissues of mesenchymal origin, through what he named "the mesengenic process"(Caplan 1994). Since then, a series of researchers have elaborated on

the use of hES for the purposes of tissue engineering and regenerative medicine (Guillot et al. 2007).

In summary the idea of utilizing stem cell transplantation for tissue regeneration or even potential organ replacement is by itself fascinating and generates a huge amount of various experimental and hypothetical approaches within the last years. Especially – similar to the principal idea of tissue engineering – the implantation of adult human autologous or embryonic stem cells, which are expanded ex vivo, might circumvent some of the current problems associated with transplantation surgery, particularly in the elderly. This encompasses the hitherto naturally limited availability of organs or tissues as well as the numerous complications that are related to disease transmission and immune rejection.

This is especially true for the complex of so called musculoskeletal degeneration, that is closely associated with the aging process. However, to introduce adult MSC into clinical practice of substituting organs or tissues, it is necessary to vigorously define the capacity of MSC to maintain growth potential and regulated differentiation of such cells into the desirable cell lineage. There is still not enough body of knowledge at the moment with regard to the physiological and pathophysiological parameters of MSC, including environmental conditions such as biomechanical forces, as to fully understand the potential influence on MSC to differentiate and grow into desired tissues, once extracted and cultured ex vivo (Cheung 2010). It is not known how MSC from young individuals behave versus cells harvested from the elderly. Nevertheless, many efforts are underways to gain more insight into the promising field of harnessing the power of stem cells for tissue and organ regeneration (Cheung 2010). Other issues that concern the ethical aspects of human embryonic stem cells need to be further addressed before research and clinical translation will make its break through.

1.4 Tissue Engineering

The origins of tissue engineering are generally traced to the beginning of the 1980s in Boston. Funding was received by the Bell Laboratories in Massachusetts Institute of Technology (MIT) for preparing a cell based vascular scaffold. Prior to that, Eugene Bell had published on the use of “living skin equivalents” in Science as early as 1981 (Bell et al. 1981). Lysaght tracked a press release in 1982 stating that “Flow General”, one of the funding firms based in Virginia, was pursuing research and development efforts in business segments including tissue engineering and “smart” computer systems (Lysaght and Crager 2009). E. Bell founded in 1985 “Organogenesis Inc.” and later on, “Tissue Engineering International – (TEI) Biosciences Inc.” both of which are renowned companies in the biotechnology landscape. During the same time, a few doors further in MIT, Joseph Vacanti of Children s Hospital approached Robert Langer with the idea to design custom made scaffolds for cell delivery. Thereupon, they started an extensive collaboration with studies on the properties of functional tissue equivalents (Vacanti 2006). In 1987 a

special session was held at the US National Science Foundation meeting in Washington DC, where the denomination "Tissue engineering" (TE) was officially given to the field and organisation of the first conference with focus on "the engineering of living tissue" was initiated, mainly by Y.C. Fung (Nerem 1992). This conference took place in 1988, at Lake Tahoe, California and the first definition of tissue engineering was introduced by Robert Nerem:

> Tissue engineering is the application of the principles and methods of engineering and the life sciences towards the fundamental understanding of structure/function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions.

The proceedings of this meeting were published a year later as a book titled "Tissue Engineering" (Skalak and Fox 1989). The first peer reviewed article accessible through the NLM database with the term appeared in 1989. It was a report on a biologically based vascular graft published by Tadashi Matsuda in ASAIO Transactions (Matsuda et al. 1989). Maybe the most cited early review on tissue engineering is a 1993 publication by JP Vacanti and R Langer in the journal Science where the definition is stated again in brief (Langer and Vacanti 1993).

1995 was a turning point of TE, since it was the year of the "auriculosaurus". Charles Vacanti, seeded a polymeric scaffold in the shape of a human ear with cartilage cells and implanted it subcutaneously on the back of a nude mouse. The pictures of this ear-formed bioartificial implant, filmed by a BBC crew, quickly made the round of the world and attracted a huge interest on the new biotechnology. It became a symbol for the emerging field of TE.

In 1996 the Tissue Engineering Society international (TESi) was officially founded by Joseph and Charles Vacanti, and the inaugural meeting took place at the Lake Buena Vista Hotel in Orlando, Florida the same year. The Asian tissue engineering societies were incorporated in TESi by 2000. By the turn of the century Raymund E Horch and G Björn Stark from Freiburg encouraged the foundation of the European branch of TESi the ETES, and they hosted in 2001 the TESi meeting in Germany.

In 1998, a clinical application of tissue engineering became popular by the media. Charles Vacanti, used a biogenic matrix out of coral seeded with osteoblasts, for reconstruction of the skeleton of a traumatized thumb (Vacanti 2006). The first tissue engineering products cleared FDA approval in the same year. Apligraf came from the E. Bell Laboratories and the firm Organogenesis as living skin equivalent. Epicel evolved from Greens laboratory whereas Yannas together with Integra Life Sciences Inc. brought in 2002 an acellular dermis regeneration scaffold by the name of Integra in the market (Kemp 2006). By the beginning of the twenty-first century, there was a wild media hype about these fascinating new technologies with unrealistic expectations both from the public and the biomedical society (Kratz and Huss 2003). Time magazine described with a cover story, tissue engineers as the "hottest job" for the future: "*With man-made skin already on the market and artificial cartilage not far behind, 25 years from now scientists expect to be pulling a pancreas out of a Petri dish*" (*What will be the 10 hottest jobs?* 2009). Just before 2001 there were over 3,000 people working in the sector, with funding exceeding US $580 million (Kemp 2006; Lysaght and Hazlehurst 2004).

1.5 The Era of Regenerative Medicine

However, by the middle of the first decade of the twenty-first century, tissue engineering seemed to be going through a crisis. Lysaght noted very graphically in 2006 that "…such highly favourable media treatment has its benefits, but research-minded professionals increasingly recognized a disconnect with the realities. And such disconnects rarely lead to happy endings" or "…Although aggregate development costs exceed $4.5 billion, the field has yet to produce a single profitable product." (Lysaght and Hazlehurst 2004).

Furthermore, tissue engineering had reached some biological limitations. The mechanistic approach dictated by biomaterial scientists, neglected issues of vascularization. It became clear that angiogenic processes were central to homeostasis, bioassimilation and biointegration of tissue engineered constructs (Mooney and Mikos 1999; Vacanti et al. 1998) Experimental activities were directed to encompass integrative strategies towards generation of autonomously vascularised bioartificial tissue elements (Polykandriotis et al. 2007, 2008) (Figs. 1.8 and 1.9). In addition to that, emphasis was being given to cellular therapies, since the era of human embryonic stem cells had arrived. Other satellite technologies had acquired a momentum of themselves, with gene technology reaching the point where a whole mammal could be easily cloned (Wilmut et al. 1997) or genetically manipulated (the Monsanto swine case). Nanotechnology came also into play with generation of new biomaterials (Beier et al. 2009).

The whole field was consecutively renamed into "regenerative medicine". The terms tissue engineering and regenerative medicine were used in parallel and synonymously to each other, but it is widely accepted that the very change of the name epitomized the beginning of a new era.

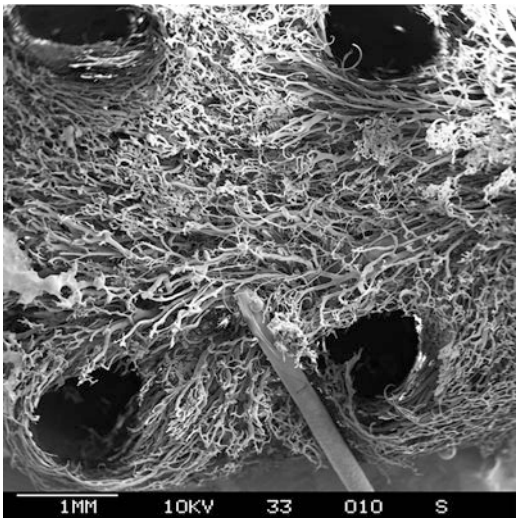


Fig. 1.8 Corrosion casting. A microvascular replica of a bioartificial organoid showing angiogenesis. The neovascular capillaries are "polarized" towards a maximum regenerative stimulus. All these capillaries were formed during the course of less than 1 week

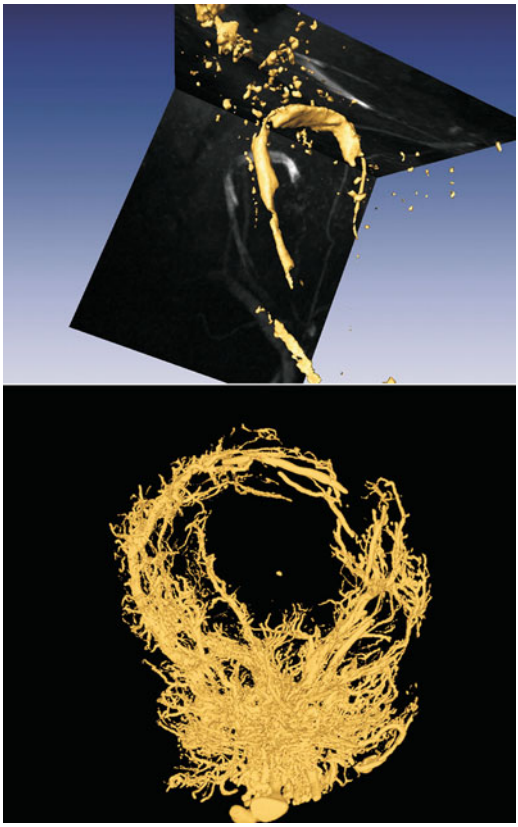


Fig. 1.9 Advanced imaging applications. *Above* Micro magnetic resonance angiography of a bioartificial organoid grown in a rat. A 4,7 Tesla Brucker bioscan equipment has been used for in vivo monitoring of the nascent biological construct. *Below* Ex vivo Micro CT study of the same organoid after injection of a contrast medium. Plasticity of the microvascular network produces a parenchyma – like circulation

The term "regenerative medicine" was popularized by William Hazeltine, the founding editor of Liebert's first e-journal Regenerative Medicine and organizer of four conferences on this same field, in Washington, DC between 2000 and 2003 (Lysaght and Crager 2009). However, he was not the first to introduce it.

The term can be found in a 1992 article on hospital administration by Leland Kaiser. In the last paragraphs there was a brief account of future technologies about to influence the sector. Under the epigram "Regenerative Medicine" it was stated that "A new branch of medicine will develop that attempts to change the course of chronic disease an in many instances will regenerate tired and failing organ systems" (Kaiser 1992). Over the next few years, several reports used the expression in connection with the New Jersey Company Integra and it could also be found in scattered newspaper reports. In spring of 1998 Business Week brought an article on biotechnology with a special reference on "Regenerative Medicine" (Arnst and Carey 1998; Lysaght and Crager 2009). The term first appears in peer-reviewed citations found on PubMed in 2000 and was in widespread use by the following year.

At the early years of the new century a shattering process took place in USA. The new legislature had frozen most of federal granting for stem cell research.

Robert Nicholas Klein II, a lawyer and real-estate developer from Palo Alto, whose son suffered from diabetes mellitus type I, and whose mother suffered from Alzheimer's disease became the leader of a public effort to change policy of the government on stem cell research and regenerative medicine. He himself invested more that $3 million from his own money on this purpose and was crowned with success. On 2 November 2004, the proposition No 71 passed through a public ballot initiative with a 59.05–40.95% majority. As a response to that, the California Institute of Regenerative Medicine was established as a superintending instance for a huge funding of more than $3 billion over 10 years. It is worth noticing, that proposition 71 was unique in many ways. First, it used General obligation bonds, which are usually used to finance construction projects such as bridges or hospitals, to fund scientific research. Second, by funding scientific research on such a large scale, California assumed a role that is typically fulfilled by the Federal Government of the United States. And third, Proposition 71 established the state constitutional right to conduct stem cell research. This initiative also represented a unique instance where the public directly decided to fund scientific research (Wikipedia 2009).

The NIH has currently adopted the following definition: "*Regenerative medicine/tissue engineering is a rapidly growing multidisciplinary field involving the life, physical and engineering sciences that seeks to develop functional cell, tissue, and organ substitutes to repair, replace or enhance biological function that has been lost due to congenital abnormalities, injury, disease, or aging*" (Daar and Greenwood 2007). The current FDA approved RM products cover a limited circumscribed range of markets in the 20–50 million scale (Kemp 2006). Still, the public and academic interest remains vivid, owing mostly to the fact that RM has the potential to tackle huge health challenges including cardiovascular disease, brain and spinal cord damage, as well as organ failure (Cheung 2010). These medical issues cost the community billions nowadays, and more often than not so, medical solutions devised so far are unsatisfactory. When biotechnological advances permit it, these medical problems will be addressed and firms well placed in the field fashioned with infrastructure, skills and a corresponding logistic network will have a clear advantage (Kemp 2006).

References

Andrews PW (1988) Human teratocarcinomas. Biochim Biophys Acta 948(1):17–36

Aristotle (1984) In: Barnes J (ed) The complete works: the revised Oxford edition, Bollingen series LXXI.2. Princeton University Press, Princeton

Arnst C, Carey J (1998) Biotech bodies. Business Week, p 56

Asenjo A (1963) Neurosurgical techniques. Charles C Thomas, Springfield

Barth A (1893) Ueber histologische Befunde nach Knochenimplantationen. Arch Klin Chir 46:409–417

Beier JP, Klumpp D, Rudisile M, Dersch R, Wendorff JH, Bleiziffer O, Arkudas A, Polykandriotis E, Horch RE, Kneser U (2009) Collagen matrices from sponge to nano: new perspectives for tissue engineering of skeletal muscle. BMC Biotechnol 9:34

Bell E, Ehrlich HP, Buttle DJ, Nakatsuji T (1981) Living tissue formed in vitro and accepted as skin-equivalent tissue of full thickness. Science 211(4486):1052–1054

BL (1794) Curious chirurgical operation – new nose. Gentlem Mag Hist Chron 64:891
California Proposition 71 (2004) Wikipedia [cited 10 Aug 2009]
Caplan AI (1994) The mesengenic process. Clin Plast Surg 21(3):429–435
Carpue J (1981(1816)) An account of two successful operations for restoring a lost nose from the integuments of the forehead. Classics of Medicine Library, Birmingham
Cheung H (ed) (2010) Stem cell and regenerative medicine. Bentham Science Publishers Ltd. [eISBN: 978-1-60805-008-6]
Coleman W (1978) Biology in the nineteenth century: problems of form, function and transformation, 2nd edn, Cambridge history of science series. Cambridge University Press, Cambridge
Daar AS, Greenwood HL (2007) A proposed definition of regenerative medicine. J Tissue Eng Regen Med 1(3):179–184
Damjanov I (1993) Teratocarcinoma: neoplastic lessons about normal embryogenesis. Int J Dev Biol 37(1):39–46
Dinsmore E (1991) A history of regeneration research: milestones in the evolution of a science. Cambridge University Press, Cambridge
Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292(5819):154–156
Goldwyn RM (1968) Johann Friedrich Dieffenbach (1794–1847). Plast Reconstr Surg 42(1):19–28
Guillot PV, Cui W, Fisk NM, Polak DJ (2007) Stem cell differentiation and expansion for clinical applications of tissue engineering. J Cell Mol Med 11(5):935–944
Hayflick L (2007) Entropy explains aging, genetic determinism explains longevity, and undefined terminology explains misunderstanding both. PLoS Genet 3(12):e220
Horch RE, Bannasch H, Stark GB (2001) Transplantation of cultured autologous keratinocytes in fibrin sealant biomatrix to resurface chronic wounds. Transplant Proc 33(1–2):642–644
Jones S (1988) Genesis. The first book of Moses, Primeval history: beginnings (1:1–1 1:32) A. Creation of the universe and of mankind (1:1–2:25) B. Canongate Books Ltd., Falkirk/ Stirlingshire. ISBN 978 0 85786 090 3
Kaiser LR (1992) The future of multihospital systems. Top Health Care Financ 18(4):32–45
Kemp P (2006) History of regenerative medicine: looking backwards to move forwards. Regen Med 1(5):653–669
Kleinsmith LJ, Pierce GB Jr (1964) Multipotentiality of single embryonal carcinoma cells. Cancer Res 24:1544–1551
Kratz G, Huss F (2003) Tissue engineering–body parts from the Petri dish. Scand J Surg 92(4):241–247
Langer R, Vacanti JP (1993) Tissue engineering. Science 260(5110):920–926
Leff D (1983) New biological assembly line. In: The cell: inter and intra-relationships. Avery Publishing Group, Wayne
Lysaght MJ, Crager J (2009) Origins. Tissue Eng Part A 15(7):1449–1450
Lysaght MJ, Hazlehurst AL (2004) Tissue engineering: the end of the beginning. Tissue Eng 10(1–2):309–320
Martin GR (1981) Isolation of a pluripotent cell line from early 29 mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78(12): 7634–7638
Mason C (2007) Regenerative medicine 2.0. Regen Med 2(1):11–18
Mason C, Dunnill P (2008) The strong financial case for regenerative medicine and the regen industry. Regen Med 3(3):351–363
Matsuda T, Akutsu T, Kira K, Matsumoto H (1989) Development of hybrid compliant graft: rapid preparative method for reconstruction of a vascular wall. ASAIO Trans 35(3):553–555
Matthews LG (1968) SS. Cosmas and Damian – Patron Saints of medicine and pharmacy their cult in England. Med Hist 12(3):281–288
Medwin T (1832) Aeschylos Prometheus bound, a tragedy. William Pickering. Printed by Charles Wittingham, Tooks Court, London

Moffatt SL, Cartwright VA, Stumpf TH (2005) Centennial review of corneal transplantation. Clin Exp Ophthalmol 33(6):642–657
Mooney DJ, Mikos AG (1999) Growing new organs. Sci Am 280(4):60–65
Nerem RM (1992) Tissue engineering in the USA. Med Biol Eng Comput 30(4):CE8 12
Newth D (1958) New (and better?) parts for old. In: Johnson M, Abercrombie M, Fogg G (eds) New biology. Penguin, London
Papaioannou VE, McBurney MW, Gardner RL, Evans MJ (1975) Fate of teratocarcinoma cells injected into early mouse embryos. Nature 258(5530):70–73
Pera MF, Cooper S, Mills J, Parrington JM (1989) Isolation and characterization of a multipotent clone of human embryonal carcinoma cells. Differentiation 42(1):10–23
Petrakova KV, Tolmacheva AA, AIa F (1963) Bone formation occurring in bone marrow transplantation in diffusion chambers. Biull Eksp Biol Med 56:87–91
Polykandriotis E, Arkudas A, Horch RE, Sturzl M, Kneser U (2007) Autonomously vascularized cellular constructs in tissue engineering: opening a new perspective for biomedical science. J Cell Mol Med 11(1):6–20
Polykandriotis E, Tjiawi J, Euler S, Arkudas A, Hess A, Brune K, Greil P, Lametschwandtner A, Horch RE, Kneser U (2008) The venous graft as an effector of early angiogenesis in a fibrin matrix. Microvasc Res 75(1):25–33
Santayana G (1905) Reason in common sense. In: The life of reason. Charles Scribner's Sons, New York, p 284
Skalak R, Fox C (1989) Tissue engineering. In: Proceedings of a workshop held at Granlibakken, Lake Tahoe, California. New York: Liss
Stocum D (2006) An overview of regenerative biology and medicine. In: Regenerative biology and medicine. Academic, Oxford, pp 1–20
Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Hearn JP (1996) Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. Biol Reprod 55(2):254–259
Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282(5391): 1145–1147
Vacanti CA (2006) The history of tissue engineering. J Cell Mol Med 10(3):569–576
Vacanti JP, Langer R, Upton J, Marler JJ (1998) Transplantation of cells in matrices for tissue regeneration. Adv Drug Deliv Rev 33(1–2):165–182
Weaver CV, Garry DJ (2008) Regenerative biology: a historical perspective and modern applications. Regen Med 3(1):63–82
What Will Be the 10 Hottest Jobs? [cited 20 Aug 2009]. Available from: http://www.time.com/time/reports/v21/work/mag_ten_hottest_jobs.html
Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385(6619):810–813
Witkowski JA (1980) Dr. Carrel's immortal cells. Med Hist 24(2):129–142
Wohlrab F, Henoch U (1988) The life and work of Carl Weigert (1845–1904) in Leipzig 1878–1885. Zentralbl Allg Pathol 134:743–751

Part I
Biology of Tissue Regeneration

Chapter 2
Extracellular Matrix and Tissue Regeneration

Yrjö T. Konttinen, Emilia Kaivosoja, Vasily Stegaev, Hanoch Daniel Wagner, Jaakko Levón, Veli-Matti Tiainen, and Zygmunt Mackiewicz

Abstract Extracellular matrix (ECM) is an important component of stem cell niche areas, which provide residence, regulate stem cell pool size and control stem cell mobilization. ECM is a complex interlinked composite of collagenous molecules, non-collagenous molecules and water-rich mucopolysaccharide ground substance. Cells are integrated to their matrix via integrin and non-integrin receptors, which are utilized in the control of adhesion, migration, division, growth, anoikis, transdifferentiation and other cellular behaviour. ECM provides architecture and strength, but also growth factor deposits, which proteinases as signalling scissors can release in a site- and process-specific manner. Several processes, like wounds, cartilage, fractures, myocardial infarctions and tumor growth are used to exemplify regenerative processes.

Y.T. Konttinen, M.D., Ph.D. (✉) • E. Kaivosoja, M.Sc. • V. Stegaev, M.D. • J. Levón, B.Sc.
Department of Medicine, Institute of Clinical Medicine, PO Box 700
(Haartmaninkatu 8, Biomedicum 1), Helsinki 00029 HUS, Finland
e-mail: yrjo.konttinen@helsinki.fi

H.D. Wagner, Ph.D.
Department of Materials and Interfaces, Weizmann
Institute of Science, Rehovot 76100, Israel

V.-M. Tiainen, Ph.D.
ORTON Orthopaedic Hospital, ORTON Foundation,
Tenholantie 10, 00280 Helsinki, Finland

Z. Mackiewicz, Ph.D., D.Sc.
Department of Histology and Embryology, Medical University of Bialystok,
G. Washington Street 13, 15-269 Bialystok, Poland

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_2,

2.1 Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) are nurtured in specialized niche areas by coordinated action via contact with soluble factors, extracellular matrix (ECM) components and stem or feeder cells, which regulate the size and mobilization of the stem cell pool. MSCs can be recruited from their protected niche locations, be locally activated, or artificially harvested from various anatomical locations to be implanted.

ECM fills the void between cells (interstitial matrix) and is present at tissue (basement membrane) and cellular (synovial lining) interfaces. ECM is a composite of fibrous (1) collagen molecules and (2) non-collagenous proteins embedded in (3) water-rich mucopolysaccharides. Small leucine-rich proteoglycans (SLRP) regulate collagen fibrillogenesis, but also cross-link fibres and cells and form deposits of growth and differentiation factors. Elastins with its associated molecules, like fibrillin and fibulin, and adhesive glycoproteins laminin and fibronectin, are important non-collagenous proteins. Large aggregating proteoglycans or lecticans, like aggrecan, and hyaluronan from the ground substance. Scaffolds or derivatization of scaffolds with ECM molecules are used to regulate proliferation and differentiation of progenitor and stem cells.

Cells are integrated to ECM via 24 different non-covalently coupled heterodimeric cellular integrin receptors, which form an important link in the integration of cellular actin cytoskeleton. They also allow exploration, migration and outside-in and inside-out signalling and act together with soluble factors and cell-cell contacts. Discoidin domain receptors, Lutheran Lu/B-CAM complex and α/β-dystroglycan complex are non-integrin matrix receptors. Glycan binding receptors, in particular extracellular C-, R- and I-type lectins and galectins play roles in cell-matrix adhesion and signalling, including MSC recruitment, bone marrow stem cell niche and adult collagen remodelling.

Proteinases modulate the composition of the ECM and are divided based on their catalytic mechanism to secretory neutral serine and metallo endoproteinases (and amino- and carboxypeptidases) and to mostly intralysosomal acidic cysteine and aspartate endoproteinases. They mediate tissue degradative events in normal remodelling and pathological tissue destruction, but are increasingly recognized as signalling scissors.

Classical examples of processes which reflect stromal stem cell function and some general and some site specific challenges comprise wound healing, cartilage healing, fracture healing, healing of myocardial infarction and tumor growth. All these are so important that intensive research is ongoing to find options for therapeutic interventions utilizing recruitment and activation of local autologous stem cells, various forms of cell therapies and tissue engineering applications.

2.2 Stem Cell Niche

Bone marrow-derived mesenchymal stem cells (MSCs) are nurtured in specialized but still poorly characterized niche areas by coordinated action via contact with soluble factors, extracellular matrix (ECM) components and stem or feeder cells.

These together regulate the size of the stem cell pool by switching them back and forth between symmetric and asymmetric cell divisions.

Healing tissue recruits cells from local already differentiated cells, which undergo symmetrical cell divisions. This recruitment extends to local resident or circulating mesenchymal stem cells (MSC) from bone marrow or other sources, which are capable of asymmetric cell divisions and able to perform various regulatory functions.

Stem cells reside in a niche, which refers to a local *in vivo* or *in vitro* cellular and matrix tissue microenvironment able to home, house, interact, maintain or mobilize one or more stem cells interacting with them and regulating their fate. The cellular "host" of the niche probably represents a cell, which is able to produce such ECM components, which the stem cell itself may not be able to produce or organize to a niche, but to which it adheres to via integrin and non-integrin matrix receptors and which ECM molecules this way regulate stem cell behaviour. Interactive participation of the nurturing "host" cell and stem cell in the process and their responsiveness to external stimuli, such as stem cell mobilizing pro-inflammatory cytokines, makes the niche dynamic. It can thus regulate stem cell proliferation and differentiation during the embryonic development but maintains stem cells in a quiescent state in adults, and helps them to get activated upon tissue injury and to disclose the potential of the stem cells to undergo also asymmetric cell divisions (Doe and Bowerman 2001). This may be determined by the orientation of the cytokinesis of the stem cell division, which is in part regulated by the architecture of the niche and by integrin mediated anchorage. If the division occurs in a plane parallel to the niche cell-stem cell contact surface, the proximally located parent cell is likely to remain in contact with the niche whereas the distally located daughter cell is displaced from it. This (1) maintains the stemness and the size of the stem cell pool and (2) produces progenitor cells, which loose contact with the niche and their stemness and leave the niche to migrate and/or transit via circulation to a new location to terminally differentiate to specialized cells, respectively (Ellis and Tanantzapf 2010). Staining of chondroitin sulphate sulphation motif epitopes can be used to identify articular cartilage progenitor cells and was used to localize perlecan and aggrecan but not versican in the stem cell niche (Hayes et al. 2008).

Interactive niche-stem cell factors can be broadly classified to three different categories, (1) soluble factors (growth factor, nutrients, electrolytes, pH, oxygen, chemokines, cytokines and differentiation factors), (2) direct cell-cell interactions (stem cell-stem cell, stem cell-feeder/other cell interactions utilizing various adhesion molecules and counter-ligands) and (3) extracellular matrix (ECM)-stem cell/niche cell interactions, which are the topic of this chapter. MSC integrin receptors for interstitial type I collagen $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$, may play a role in this respect, but hMSC have also been described to contain e.g. α_3, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (for more, see Docheva et al. 2007), which may help the stem cells to home, anchor, structure, divide and leave the niche as well as otherwise to perform their other functions as stem cells. However, in spite of the knowledge of the integrins present in hMSCs, the actual integrin receptors relevant for the niche cells and for the MSCs are poorly known at present, but based on currently available

information on different stem cell-niche systems, several redundant integrin receptors are probably involved (Ellis and Tanantzapf 2010).

Integrin chain specific antibodies coupled to paramagnetic micro-beads, which are subjected to an inhomogeneous magnetic field, and various computer controlled culture substrate stretching devices have been developed to test the effect of integrin-mediated mechanical forces on cellular phenotype and function (Sasaki et al. 2007; Pommerenke et al. 1996). Mechanotransduction may help to shape the stem cell niche and regulate the stem cell function as has been recently proven by mechanoactive tenogenic differentiation (Kuo and Tuan 2008).

Understanding the effects of the above mentioned niche factors on chromatin remodelling (heterochromatin or active euchromatin state) and gene expression, (including microRNA-mediated epigenetic regulation), is essential for proper control of tissue engineering constructs produced in cell culture, bioreactors or *in situ* utilizing heterotopically seeded cells or cells locally recruited to the scaffold or damage area. A delicate balance protects stem cells from depletion, but at the same time prevents excessive, cancer cell-like proliferation. Stem cell niche constitutes the basic unit of stem cell physiology the same way as osteons, chondrons and salivons do in bone, cartilage and salivary gland tissue, respectively. Integration of signals at the interface between the bone marrow and stem cells in the niches are still poorly understood.

Niche has relatively wide implications also for cancer cells and hematopoietic cells. Self-renewing cancer stem cells may reside and renew in cancer stem cell niche composed of a specialized vascular bed of endothelial cells, some sort of mesenchymal cells and ECM components (Nie 2010). Bone marrow derived MSCs in their bone marrow stem cell niche may via their immunosuppressive properties be involved in cancer progression and metastasis. MSCs of bone marrow may also provide the cellular support structure in the niche for hematopoietic stem cells (Battiwalla and Hematti 2009).

In spite of their predicted existence, it is a challenge to learn to unanimously identify stem cells in their natural surrounding. In tissues they can not be recognized by their ability to differentiate along various specialized cell lineages and demonstration of a palette of markers used for cultured or cloned stem cells by using flow cytometry are not easily adaptable to histological tissue sections at the single cell level in static cytometry.

To regulate the size of the stem cell or progenitor cell pool, stem cells have a capability pendulate between the above mentioned asymmetric and symmetric cellular divisions to either increase the stem cell pool or the progenitor pool. Probably the stem cell pool is expanded during embryogenesis, whereas asymmetric divisions could allow rapid generation of progenitors upon high demand in various tissue regenerative processes. The process of expansion of the stem cell pool must be controlled at some critical checkpoints to prevent cancer, whereas to latter is also strictly regulated to prevent precocious depletion of the stem cell pool; the cells can probably switch back and forth between these two different modes of proliferation. The size of the stem cell pool is probably downsized upon aging via senescence or apoptosis.

2.3 Mesenchymal Stromal Cells

Mesenchymal stem cells can be recruited from their protected niche, locally activated, or artificially harvested from various anatomical locations to be implanted as such or in tissue engineering constructs to tissue defects to facilitate repair by expansion, differentiation or perhaps mostly by orchestration of the more simply programmed resident or immigrant repair cells during tissue regeneration.

Bone, cartilage and other tissue defects can be repaired by differentiated autologous cells or tissues isolated from the donor site(s) for transplantation, but often it is impossible to obtain enough autologous cells or tissues for such repair procedures and harvesting them from e.g. iliac bone or non-weight bearing femoral head cartilage can be complicated and painful. Usage of autologous cells requires usually enrichment and control of phenotype. Allogeneic and xenogeneic cells would be better available for clinical use, but immunosuppressive treatment would be necessary to overcome immunological rejection evoked by foreign cells. Only scid mice with severe combined immunodeficiency can be used for experimental studies of such allogeneic or xenogeneic cells. Foetal cells might provide a low immunogenic option (O'Donoghue and Fisk 2004) but the use of foetal and embryonic stem cells raises ethical issues.

Tissue injury triggers cellular mechanisms, which lead to injury site specific cell division and matrix synthesis, which regulate homing and engraftment of circulating and local stem cells to the void and to regulate the sequential and ordered healing cascades. To avoid scar tissue formation and promote true regeneration with functional tissue with original tissue characteristics, MSCs and various natural or synthetic scaffolds seeded with mesenchymal (or even foetal or embryonic) stem cells have raised interest. They could be used to augment healing in critical size defects, non-union, non-healing and otherwise clinically threatening tissue defects.

Autologous MSCs from various sources in adults have potential to expand in numbers via symmetric cell divisions and then to differentiate into different specialized phenotypes via asymmetric cell divisions. The optimal source of stem cells for bone, cartilage and tissue engineering purposes is still debated due to insufficient knowledge on the eventual commitment of cells of different origins. Potential sources include bone marrow, adipose tissue, synovial membrane, skeletal muscle, periosteum, dental pulp, umbilical cord blood, amniotic fluid and others.

Apart from differentiation cocktails containing mixtures of somewhat variable growth and differentiation factors and basic nutrient medium or transduction of several genes to already differentiated cells such as skin fibroblasts to produce induced progenitor cells, also several other actors play a role. ECM-integrin interface exerts mechanotransductive influences on MSCs so that mechanical stimulation may commit them towards osteogenic lineage (Engler et al. 2006), although excessive mechanical strain may again favour fibroblast-like phenotype, e.g. at the implant-bone interface or tendon formation as described above. Apart from mechanical forces, hypoxia is likely to play a role in the differentiation of MSCs to bone, fibrous tissue, cartilage or fibrocartilage, the formation of the latter two being favoured by hypoxia.

Vision of MSCs as precursors for resident cellular components of various specialized tissues is oversimplified, because MSCs can prolong the survival of skin and cardiac allografts, ameliorate acute graft-versus-host disease and experimental autoimmune encephalomyelitis, which all indicate important regulatory roles in the orchestration of specialized local cells in void filling tissue repair (Zhao et al. 2010; Keating 2012).

2.4 Extracellular Matrix

Extracellular matrix fills the void between cells (interstitial matrix) and is present at tissue (basement membrane) and cellular (synovial lining) interfaces. It is synthesized, maintained and modulated by cells to adapt to development, growth, aging, changing mechanical and developmental needs, to meet the needs after micro- and macrodamage to renew and regenerate and to produce new editions of instructive outside-in signalling matrix. ECM is a composite of fibrous (1) collagen molecules and (2) non-collagenous proteins embedded in (3) water-rich mucopolysaccharides. Small leucine-rich proteoglycans (SLRP) regulate collagen fibrillogenesis (fibre thickness), but also cross-link fibres and cells and form deposits of growth and differentiation factors. Elastin with its associated molecules, like fibrillin and fibulin, and adhesive glycoproteins laminin and fibronectin, are important non-collagenous proteins. Apart from SLRPs, large aggregating proteoglycans or lecticans, like aggrecan, and hyaluronan from important components of the ground substance. Scaffolds or derivatization of scaffolds with ECM molecules are used to regulate proliferation and differentiation of progenitor and stem cells.

2.4.1 Extracellular Matrix: Collagens

ECM fills the void between cells (interstitial matrix) and between tissue interfaces (basement membrane), providing by its toughness structure and physical support to tissue-typical multicellular but dynamically generated (organogenesis) and maintained (remodelling) architecture as well as adhesion substrate and an instructive editable matrix, which literarily is decisive for cellular survival and for the regulation of multiple aspects of cellular behaviour (Aszódi et al. 2006). For most soft (skin, fat, fasciae, muscles, tendons, blood vessels, brain, peripheral nerves etc.) and hard or semi-hard (bone, cartilage, cornea etc.) “connective tissues” collagen nanofibres form a three dimensional and highly organized scaffolded backbone, whereas the more hydrophilic and permeable ground substance largely composed of hydrophilic proteoglycans and glycoproteins occupies the interfibrillar spaces enabling transfer and filtration of nutrients, oxygen and metabolites. Linker proteins bind these two major components to extensive networks, which provide dynamic and adjustable biomechanical strength, associated with flexibility, to such cell-matrix composite structures. Due to the high biomechanical and instructive demands imposed to the

ECM, it undergoes almost continuous mechanotransduction and remodelling by replacing damaged and degenerated tissue with new intact and properly organized tissue to adapt to the varying local functional needs (Aszódi et al. 2006).

Human body contains altogether 28 different collagen types, which are classified to nine different subtypes, including fibrillar collagens, which form the bulk of the interstitial stromal collagens as described above and in some more detail in Table 2.1. Structurally all collagens are characterized by the archetypical Gly-X-Y repeat sequences, in which X is often proline and Y often either hydroxyproline or hydroxylysine the formation of which requires specific hydroxylases and vitamin C. After synthesis of collagen α-chains, regularly repeated glycins with their minimal side chains (–H) allow three individual collagen chains to wind up around each other into triple helical collagen monomer, procollagen, with globular amino- and carboxyterminal propeptide heads. The collagen superhelix domain is, due to its structure, very resistant against non-specific proteinase-mediated degradation. After processing and removal of the globular propeptides individual collagen monomers, tropocollagens, spontaneously non-enzymatically assemble side by side to near one quarter overlapping supramolecular stacks with the typical cross-striation visible in electron micrographs developing as a result of this (fibrillogenesis). Fibre thickness is for type I collagen regulated in part by type III collagen and for type II collagen in part by collagens IX (embedded in part inside the collagen type II fibre) and XI (located on the surface of collagen type II fibre). These still relatively loose fibre stacks mature by covalent cross-linking in a process, which involves specific cross-linking enzymes, lysyl oxidase. Collagen network provides substrate for attachment of ground substance and cells and provides the framework for deposition of various bioactive factors.

Purified native or processed allogeneic and recombinant human collagens, especially type I, are composed of nanosize biodegradable biofibres with potential for use in plastic and cosmetic surgery, drug delivery and tissue engineering in form of sheets, pellets, plugs, sponges and other products. Collagen sponge seeded with bone marrow-derived MSCs can develop healing tissue which to its biomechanical strength to 75% matches that of the corresponding healthy tissues (Juncosa-Melvin et al. 2006).

Basement membranes support epithelia and endothelia, casting them to their structural shapes, such as simple sheets or tubes or relatively sophisticated tubuloacinar, tubuloalveolar and vascular structures. Basement membrane also surrounds some individual cells, like adipocytes, Schwann cells and skeletal muscle cells. Basement membrane components are found in the intercellular cementing substance between fibroblast-like type B and macrophage-like type A synovial lining cells joining them to form synovial lining or intima of the joint cavity, which in spite of its sheet-like structure does not have an actual sheet-like basement membrane.

Basement membrane is a 100–300 nm thick barrier with perforations in the order of ~50 nm permitting free bidirectional movement of small molecules whereas the movement of cells and larger molecules is controlled. Certain cells, like the neutrophils, do cross the BM with great efficiency. Chemoattractants and proteolytic events play a role in this process.

Table 2.1 Classification and types of collagens based on the collagen chain encoding genes, their distribution in tissues and disorders caused by their mutations

Classification	Type	Own chains	Gene	Exons	Chromosome[a]	Distribution in Tissues	Disorders Caused by Mutation in Genes
Fibrillar collagens	I	α_1	*COL1A1*	51	17q21.3-q22	Bone, tendon, ligament, skin	Osteogenesis imperfecta, osteoporosis
		α_2	*COL1A2*	52	7q22.1		
	II	α_1	*COL2A1*	54	12q13.11	Cartilage, intervertebral disc, vitreous humor	Several chondrodysplasias, osteoarthritis
			Col2a1	54			
	III	α^1	*COL3A1*	51	2q24.3-q31	Co-expressed with collagen I in vasculature and skin	Ehlers-Danlos syndrome (type IV), arterial aneurysms
			Col3a1	51	1		
	V	α^1	*COL5A1*	66	9q34.2-q34.3	Co-expressed with collagen I in lung, cornea and bone	Ehlers-Danlos syndrome (types I and II)
		α_2	*COL5A2*	54	2q14-q32		
		α_3	*COL5A3*	66	19p13.2		
	XI	α_1	*COL11A1*	68	1p21	Co-expressed with collagen II	Chondrodispasias, non-systematic heating loss, osteoarthritis
		α_2	*COL11A2*	66	6p21.2		
		α_1(II)					
	XXIV	α_1	*COL24A1*	57	1p22.3	Co-expressed with collagen I in bone and cornea	Not known
	XXVII	α_1	*COL27A1*	61	9q32	Co-expressed with collagen II in cartilage and epithelia	Not known
	XXVIII	α_1	*COL28A1*	32	7p21.3	Peripheral nerves	Not known
3D network (BM-collagens)	IV	α_1	*COL4A1*	52	13q34	Most basement membranes	Alport syndrome
		α_2	*COL4A2*	47	13q34	Glomerular and alveolar BM	(COL4A3, COL4A4, COL4A5)
		α_3	*Col4a2*	47	8		Alport syndrome with diffuse oesophageal leiomyomatosis (COL4A5, COL4A6)
		α_4	*COL4A3*	52	2q34-q37		Lethality at 14 weeks, progressive glomerulonephritis, renal failure[b]
		α_5	*COL4A4*	48	2q35-q37		
		α_6	*COL4A5*	51	Xq22		
			COL4A6	46	Xq22		

Microfibril (beaded-filaments)	VI	α_1,	*COL6A1*	36	21q22.3	Wide tissue distribution, not bone	Bethlem myopathy
		α_2,	*COL6A2*	36	21q22.3		
		α_3	*COL6A3*	41	2q37		
Anchoring fibril	VII	α_1	*COL7A1*	118	3p21	Squamous epithelium BM zone	Epidermolysis bullosa
			Col7a1	118	9		
Hexagonal lattice	VIII	α_1,	*COL8A1*	5	3q12.3	Many tissues, Descement's membrane of cornea	Corneal endothelial dystrophy
		α_2	*COL8A2*	2	1p34.2		
	X	α_1	*COL10A1*	3	6q21-q22	Hypertrophic cartilage	Schmid metaphyseal chondrodisplasia
			Col10a1	3	10		
FACITs	IX	α_1,	*COL9A1*	38	6q12-q14	Associated with type II fibrils in cartilage and cornea	Epiphyseal dysplasia, intervertebral disc disease, osteoarthrosis
		α_2,	*COL9A2*	32	1p32		
		α_3	*Col9a2*	32	4		
			COL9A3	32	20q13.3		
	XII	α_1	*COL12A1*	65	6q12-q13	Associated with type I fibrils in perichondrium, ligament, and tendon	Disruption of periodontal and skin matrix structure[b]
	XIV	α_1	*COL14A1*	44	8q23	Associated with type I fibrils in many tissues	Not known
	XVI	α_1	*COL16A1*	67	1p35-p34	Associated with type II fibrils in hyaline cartilage and with microfibrils in skin	Not known
	XX	α_1	*COL20A1*	35	20q13.33	Associated with type I fibrils in sternal cartilage, cornea, and tendon	Not known
	XXI	α_1	*COL21A1*	28	6p12.3-p11.2	Associated with type I fibrils in vessel walls	Not known

(continued)

Table 2.1 (continued)

Classification	Type	Own chains	Gene	Exons	Chromosome[a]	Distribution in Tissues	Disorders Caused by Mutation in Genes
FACIT-like	XIX	α_1	*COL19A1*	51	6q12-q14	Rare BM zones, in developing muscle	Abnormal muscle layer in the oesophagus[b]
	XXII	α_1	*COL22A1*	63	8q24.23	Associated with microfibrils at tissue junctions	Not known
	XXVI	α_1	*EMID2*	13	7q22.1	Testis and ovary	Not known
Trans-membrane collagens	XIII	α_1	*COL13A1*	41/42	10q22	Many tissues at a low level	Fetal lethal, cardiovascular and placental defects, tumor formation[b]
			Col13a1	42	10		Progressive muscular atrophy[b]
	XVII	α_1	*COL17A1*	56	10q24.3	Skin and intestinal epithelia	Epidermolysis bullosa
	XXIII	α_1	*COL23A1*	20	5q35.3	Heart, lung and brain, m etastatic tumor cells	Not known
	XXV	α_1	*COL25A1*	35	4q25	Neurons	Not known
Multiplexins	XV	α_1	*COL15A1*	42	9q21-q22	Many BM zones	Mild myopathy, cardiovascular defects[b]
			Col15a1	40	4		
	XVIII	α_1	*COL18A1*	43	21q22.3	Endothelial and epithelial BM zones	Knobloch syndrome
			Col18a1	43	10		Vascular abnormalities in the eye[b]

BM basement membrane

[a]The cromosomal locations and the exones were collected from the Entrez Gene data base

[b]In transgenic mouse models; Modified from Jäälinoja (2007), Cosgrove et al. (1996), Reichenberger et al. (2000), Myllyharju and Kivirikko (2001), Fukai et al. (2002), Sund et al. (2001), Kvist et al. (2001), and Eklund et al. (2001)

It has been thought that type IV collagen polymer network serves as the base platform upon which that laminin network is deposited but laminin polymers may actually serve as a template for the subsequent assembly of the BM (Li et al. 2005a; McKee et al. 2007). Collagen IV and laminin forms ternary complexes linked together by nidogen-1 and -2 (Fox et al. 1991; Kohfeldt et al. 1998). A heparan sulphate/chondroitin sulphate proteoglycan, perlecan, is also found as an integral part of this network and is important for its integrity and as a local storage of growth factors (Gohring et al. 1998).

The type IV collagens were first identified by Kefalides in 1966 (Kefalides 1966). Depending upon its location it is synthesized either by fibroblasts, paranchymal cells, epithelial cells, endothelial cells, or by various other cells that are surrounded by the BM. The collagen type IV genes in human encoding its different α chains are arranged head-to-head in three pairs. Gene encoding type IV collagen α_1, α_2 (*COL4A1- COL4A2*) and α_3, α_4 (*COL4A3-COL4A4*) is located on chromosome 13, and on chromosome 2, respectively, while the gene encoding for type IV collagen α_5, α_6 (*COL4A5-COL4A6*) is located on chromosome X. A common ancestral gene may have been duplicated three times resulting in six evolutionary related genes (Zhou et al. 1994). Sequences and characteristic exon–intron organizations divide them into α_1-like group (*COL4A1*, *COL4A3*, *COL4A5*), and α_2-like group (*COL4A2*, *COL4A4*, *COL4A6*). A unique feature of the type collagen IV gene pairs is that they share bidirectional promoters.

All type IV collagen α chains are ~1,400 amino acids long. They are composed of a ~15-residues long N-terminal 7S domain, collagenous segments consisting of Gly-X-Y repeats, which are interrupted by 22 short non-collagenous sequences (which provide flexibility and serve as cell-binding sites) and a ~230-residue long C-terminal non-collagenous NC1 domain (Kalluri 2003). Three type IV collagen α wind up to a triple helical tropocollagen. According to the currently known combinatorial rules three distinct trimers are formed, $\alpha_1\alpha_1\alpha_2$, $\alpha_3\alpha_4\alpha_5$, and $\alpha_5\alpha_5\alpha_6$ (Boutaud et al. 2000; Hudson et al. 2003; Khoshnoodi et al. 2008).

The collagen monomers further self-assemble to supramolecular networks. First, dimers are formed by head-to-head association of two protomers via their trimeric NC1 domains so that bonding NC1 hexamers are formed. Four dimers then join at their N-terminal cystine- (disulfide bonds) and lysine-rich (lysine-hydroxylysine bonds) containing regions to form a heavily glycosylated 7S-tetramer. This knot is relatively resistant to collagenase activity and can be isolated from bacterial collagenase treated basement membranes at a sedimentation coefficient 7S (Risteli et al. 1980). $\alpha_1\alpha_1\alpha_2$(IV) and $\alpha_3\alpha_4\alpha_5$(IV) trimers form independent networks, while $\alpha_1\alpha_1\alpha_2/\alpha_5\alpha_5\alpha_6$ (IV) molecules form combined aggregates (Borza et al. 2001).

Type IV collagen composition of the basement membrane seems to affect cell adhesion, proteolytic susceptibility and ability to interact with other BM components (Kalluri 2003). The α_1(IV) and α_2(IV) are ubiquitously found throughout the human body, while the other 4 α chains have a more restricted tissue distribution. The $\alpha_3\alpha_4\alpha_5$(IV) network is found in the glomerular and some tubular basement membranes of the kidney, cochlea, eye, lung and testis, whereas the $\alpha_5\alpha_5\alpha_6$(IV) trimer is located in the skin, oesophagus, Bowman's capsule of the kidney and

smooth muscle cells. A temporal regulation of type IV collagen α chains expression is seen for instance in the glomelular BM of human kidney. During early embryonic development (day 75), the genes which encode α_1(IV) and α_2(IV) chains are expressed. As the development proceeds the expression of genes encoding the α_3(IV), α_4(IV) and α_5(IV) chains starts while the levels of α_1(IV) and α_2(IV) chains gradually decrease. This switch in gene expression during developmental is critical for maturation of the glomerular BM.

Mutations in the genes encoding either α_1(IV) or α_2(IV) are embryonic lethal, while mutations in the genes encoding the α_3(IV), α_4(IV), α_5(IV) or α_6 (IV) chains may lead to human diseases (Hudson et al. 2003; Hudson 2004) (Table 2.1). Mutation of the *COL4A5* gene results in Alport syndrome. This mutation is mostly inherited and results in glomerulonephritis and hearing loss. Goodpasture's syndrome is an autoimmune disease manifest by rapidly progressive glomerulonephritis and pulmonary hemorrhage. The Goodpasture antigen, which is usually the NC1 domain of the α_3(IV) chain, is most exposed in the glomeruli and alveolar basement membranes.

Type IV collagen expression is suppressed by pioglitazone (Ohga et al. 2007; Ko et al. 2008) and methotrexate (Yozai et al. 2005). Tumstatin, the NC1 domain of α_3(IV) chain, is an endogenous inhibitor of pathological angiogenesis and suppresses tumour growth via integrin $\alpha_v\beta_3$, because tumstatin binding inhibits focal adhesion kinase and some other signalling pathways, which inhibits endothelial cell proliferation and induces apoptosis (Maeshima et al. 2001, 2002).

Endothelial cells, basal cell layer keratinocytes, hepatocytes, carcinoma cells, melanoma cells, fibrosacroma cells and many other cells bind via adhesion receptors to multiple sites in the NC1 and/or the triple helix domains of type IV collagen as explained below in the paragraph on integrin and non-integrin receptors.

2.4.2 Extracellular Matrix: Non-Collagenous Proteins

Elastin is a highly elastic stretchable and recoiling strong and elastic fibrous protein of many connective tissue matrices of the body, including in particular large and medium size arteries (conductance vessels, e.g. aorta), lung alveoli, skin and intervertebral discs. It yields under stress but stores energy upon stretching. Polymorphic and soluble tropoelastin monomers is produced and secreted by smooth muscles cells in arteries and by fibroblasts. Around 65 kD size, glycine-, proline-, valine- and alanine-rich tropoelastin monomers are rapidly close to their cellular source covalently cross-linked by lysine oxidase to form elastic di-, tri- or tetrafunctional crosslinks, e.g. desmosine, isodesmosine. This leads to the formation of irregularly organized and randomly coiled amorphous and yellowish elastin-rich networks and sheets. In these structures elastin is surrounded by fibulin and fibrillin sheaths and a pathogenic mutation of fibrillin-1 is linked to Marfan syndrome with e.g. risk for dissection of the aorta.

Heterotrimeric laminins glycoporteins form one of the two major non-collagenous networks in the basement membranes. The laminin network is via entacin or

Table 2.2 Classification laminins, their abbreviations according to current nomenclature with some alternative names and the genes encoding them

Laminin (LM)	Abbreviation and alternative names	Genes encoding the laminin chains
LM-$\alpha_1\beta_1\gamma_1$	LM-111, Ln-1	*LAMA1, LAMB1, LMAC1*
LM-$\alpha_2\beta_1\gamma_1$	LM-211, Ln-2	*LAMA2, LAMB1, LAMC1*
LM-$\alpha_1\beta_2\gamma_1$	LM-121, Ln-3	*LAMA1, LAMB2, LAMC1*
LM-$\alpha_2\beta_2\gamma_1$	LM-221, Ln-4	*LAMA2, LAMB2, LAMC1*
LM-$\alpha_3A\beta_3\gamma_2$	LM-332/LM-3A32, Ln-5/5A	*LAMA3A, LAMB3, LAMC2*
LM-$\alpha_3B\beta_3\gamma_2$	LM-3B32, Ln-5B	*LAMA3B, LAMB3, LAMC2*
LM-$\alpha_3A\beta_1\gamma_1$	LM-311/LM-3A11, Ln-6	*LAMA3A, LAMB1, LAMC1*
LM-$\alpha_3A\beta_2\gamma_1$	LM-321/LM-3A21, Ln-7	*LAMA3A, LAMB2, LAMC1*
LM-$\alpha_4\beta_1\gamma_1$	LM-411, Ln-8	*LAMA4, LAMB1, LAMC1*
LM-$\alpha_4\beta_2\gamma_1$	LM-421, Ln-9	*LAMA4, LAMB2, LAMC1*
LM-$\alpha_5\beta_1\gamma_1$	LM-511, Ln-10	*LAMA5, LAMB1, LAMC1*
LM-$\alpha_5\beta_2\gamma_1$	LM-521, Ln-11	*LAMA5, LAMB2, LAMC1*
LM-$\alpha_2\beta_1\gamma_3$	LM-213, Ln-12	*LAMA2, LAMB1, LAMC3*
LM-$\alpha_3\beta_2\gamma_3$	LM-323, Ln-13	*LAMA3, LAMB2, LAMC3*
LM-$\alpha_4\beta_2\gamma_3$	LM-423, Ln-14	*LAMA4, LAMB2, LAMC3*
LM-$\alpha_5\beta_2\gamma_3$	LM-523, Ln-15	*LAMA5,LAMB2, LAMC3*
LM-$\alpha_5\beta_2\gamma_2$	LM-522	*LAMA5, LAMB2n, LAMC2*

Modified from Patarroyo et al. (2002), Aumailley et al. (2005), Tzu and Marinkovich (2008), and Egles et al. (2007)

nidogen linked to the other major basement membrane network, which is composed of type IV collagen. Laminins are composed of five different α chains (of which α_3 chain has two variants), three different β chains and three different γ chains, which according to currently known combinatorial rules can combine to 17 different trimeric laminin molecules (Table 2.2). They have a tightly regulated tissue-specific localizations to be able to contribute to the heterogeneity and site-specific regulation of cells and tissues (Table 2.3). This latter aspect should be emphasized, because in spite of the fact that the effect of soluble regulatory factors can be confined in space and time by localized synthesis, deposition and release and short half life and thus short range of action, they still can by accident diffuse over and beyond their physiological limits to cause pathology, whereas solid regulatory molecules are from this point of view more site specific and safe.

Laminin α_1 in the acinar basement membrane in salivary glands is an example of a site-specific factor, which can help migration of progenitor cells from the adjoining intercalated duct to the acinar space and then guides locally trans-differentiation of the progenitor to a mature acinar cell capable of exocrine secretion. This process seems to proceed normally in salivons in healthy human salivary glands, but to be disturbed in a common salivary gland disease known as Sjögren's syndrome, which syndrome is characterized by qualitative and quantitative impairment of exocrine secretion (Laine et al. 2004, 2008). This laminin α_1-guided (according to the combinatorial rules of Table 2.2 a component of LM-111 composed of laminin α_1, β_1 and γ_1 chains) and Int $\alpha_1\beta_1$ and $\alpha_2\beta_1$-mediated process can also be reproduced in an *in vitro* model utilizing

Table 2.3 Tissue distribution of laminin alpha, beta and gamma chains

α_1	Early embryo, Neuroretina, Adult kidney proximal tubules, Salivary and mammary glands
α_2	Trophoblast, foetal skin and kidney, adult skin, skeletal and cardiac muscle, peripheral nerve, some capillaries, brain and other tissues
α_3	Foetal skin, lung alveoli and bronchi, adult skin, alveoli, bronchi and most other epithelia
α_4	Foetal skin and kidney, skeletal muscle, adult skin, cardiac and visceral smooth muscles, nerve, blood vessel endothelia, bone marrow and other tissues
α_5	Foetal skin, lungs and kidney, adult skin, alveoli, bronchi, diverse epithelia, kidney, blood vessels, bone marrow, developing muscle and nerve, synaptic membrane
β_1	Most tissues
β_2	Foetal bronchi and alveoli, kidney, adult neuromuscular junction, blood vessels, kidney glomeruli
β_3	Foetal skin and lungs, adult skin and most other epithelia
γ_1	Most tissues
γ_2	Foetal skin and lungs, adult skin, bronchi and most other epithelia
γ_3	Kidney, lungs, reproductive tract, nerve and brain

Modified from Patarroyo et al. (2002) and Tzu and Marinkovich (2008)

human submandibular gland HSG cells of an intercalated duct phenotype. Upon culture on and in laminin α_1 chain containing Matrigel they trans-differentiate to acinar cells without any need for support by any additional growth and differentiation factors. Thus, laminins affect tissue morphogenesis, maintenance and function by influencing proliferation, migration and differentiation (Jones et al. 2000).

One important adhesive fibrous glycoprotein either locally synthesized or precipitated from the circulation is fibronectin. In spite of local fibroblast-mediated fibronectin synthesis, fibronectin does not stain or stains only weakly at the base of chronic, non-healing ulcers (Herrick et al. 1992, 1996). This is probably due to rapid proteolytic degradation of newly synthesized extracellular fibronectin matrix in such inflammatory and proteinase-rich environment (Weckroth et al. 1996). Cartilage oligomeric matrix protein (COMP) is a pentamer with five collagen binding "arms", found in cartilage, ligaments and tendons. COMP binds to free collagen type II and I molecules facilitating formation of banded fibres. It is not found in mature fibres, except at the tip/end of eventually growing fibres.

2.4.3 Extracellular Matrix: Ground Substance

Ground substance is largely composed of proteoglycans, which are composed of an organizing protein core on the surface of the cell or in the interstitium, with attached linear hydrophilic glycosaminoglycan (GAG) bipolymers (mucopolysaccharides). GAGs are composed of 50–1,000 repeat disaccharide units and based on the structure of the disaccharide backbone, chemical bonding utilized between the sugar residues and side chain modifications (such as acetylation and sulphation).

GAGs are divided into (1) heparin composed of L-iduronate α(1→4) N-sulfo-D-glucosamine-6-sulphate α(1→4) backbone with variable degrees of sulphation of the L-iduronate (2-O position) and/or glucosamine (3-O or 6-O position, in addition, the N-position of the glucosamine can be sulphated, acetylated or unsubstituted, located in mast cell granules), (2) heparan sulphate composed of D-glucuronate β(1–3) N-sulfo-D-glucosamine-6-sulfate β(1–4) with variable degrees of sulphation of the glucoronate (2-O position) and/or N-acetylglucosamine (3-O or 6-O position, in addition the N-position of the glucosamine can be acetylated, sulphated or unsubstituted). It contains fewer N- and O-sulphate groups and more N-acetyl groups than heparin, but it is heterogenous as it also contains heparin-like segments, found e.g. in cell surface proteins, lung, basement membranes, heparin or heparin sulphate are found in extracellular perlecan (can alternatively contain chondroitin sulphate), cell surface syndecans and glypicans and a small leucin-rich proteoglycan (SLRP) known as prolargin (coded by the *PRELP* gene, standing for proline arginine-rich end leucine-rich repeat protein); SLRPs may in addition to proteoglycans also contain O-linked oligosaccharides and sulphated tyrosine residues, and one member, integrin-binding chondroadherin, only contains O-linked short oligosaccharides, which form only 1% of its molecular mass, (3) chondroitin sulphate (composed D-glucoronate β(1–3) N-acetyl-D-galactosamine β(1–4) backbone with variable degrees of sulphation of the glucuronate (carbon 2) and/or N-acetyl-D-galactosamine (carbon 4 in chondroitin-4-sulphate and/or carbon 6 in chondroitin-6-sulphate), e.g. cartilage, bone, tendons, ligaments, found in large aggregating proteoglycans or hyaluronan-binding lecticans (hyalectans), like aggrecan (forming 95% of the proteoglycans in cartilage, bound to hyaluronan core), versican, neurocan and brevican, and some SLRP which contain either chondroitin and/or dermatan sulphate side chains, as found in decorin/small proteoglycan II (1 chain), biglycan/small proteoglycan I (2 chains) and epiphycan (2 chains) in the epiphysis), (4) dermatan sulphate (differs from chondroitin sulphate by also containing L-iduronate α(1→3) N-acetyl-D-galactosamine-4-sulphate β(1→4) disaccharides in its backbone with variable degrees of sulphation of the iduronate (carbon 2) and/or N-acetyl-D-galactosamine (carbon 4 and/or 6), e.g. skin, blood vessels, heart valves) and (5) keratan sulphates (composed of D-galactose β(1→4) N-acetyl-D-glucosamine-6-sulphate β(1→3) with variable degrees of sulphation of the galactose (carbon 6) and/or N-acetyl-D-glucosamine (carbon 6), e.g. cornea, bone, cartilage, nucleus pulposus, found in some SLRPs, like lumican, keratocan and mimecan (osteoglycin or osteoinductive factor) in the transparent cornea, integrin-binding osteoadherin (osteomodulin) in mineralized tissues and fibromodulin in the cartilage, all with 1–3 N-linked keratan sulphate chains and sulphated tyrosine residues). The sulphate content is highly variable and its molecular components are occasionally substituted with e.g. fucose or mannose. For hyaluronan, see below.

At the physiological pH most of the sulphate and carboxyl groups of these long molecules are negatively charged making these molecules viscous, highly charged, able to bind water and elastic. These molecules exert swelling pressure checked by the collagen fibres of the matrix. These proteoglycans occur as cell surface and interstitial molecules and provide a cell-friendly hydrogel-like but permeable

surrounding for the cells. Perhaps the best recognized role these proteoglycans relates to their ability to bind and deposit growth factors, like most of the 22 now known fibroblast growth factors (FGFs, some of these bind less avidly and can have systemic, endocrine actions), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), vascular endothelial growth factors (VEGF) and endostatin derived from degradation of type XVIII collagen (with anti-angiogenic properties). Due to local release and paracrine mode of action these GAG-deposited growth regulating factors play important roles in tissue repair. Chondroitin sulphate sulphation motif epitopes are useful in the identification of articular cartilage progenitor cells.

Some hundred bottle brush-like aggrecan molecules, the prototype of lecticans, are in cartilage attached to a hyaluronan core via the globular G1 domain of the aggrecan core molecule located at the N-terminus. This binding to hyaluronan is enforced by a HA-binding link protein. G1 is via an interglobular domain combined with another globular domain, G2, followed by a long GAG-binding region, first one keratan sulphate-rich region (able to bind collagen) and then two chondroitin sulphate-rich regions, and finally a third globular domain G3 (composed of a splice-dependent complement regulatory protein-like module and an epidermal growth factor (EGF)-like modules, and a constant C-type lectin-module) able to mediate binding to fibulin-1 and-2, fibrillin-1 and tenascin-R, but according to its lectin nature also to cell surface glycolipids. Several of the G3 domain ligands are di- or oligomers and could therefore mediate cross-linking of the hyaluronan-lectican complexes to each other. Such cross-linking would be impaired upon age-related fragmentation of aggrecan and loss of the cross-linking G3 domains. Early arthritis is characterized by loss of metachromatic proteoglycan staining, which is due to proteolytic solubilisation of aggrecan by ADAMTS4 (a disintegrin and a metallo-proteinase with a thrombospondin motif 4) and ADAMTS 5, often at the sensitive interglobular domain. Versican is named for its versatile molecular structure, is produced by vascular smooth muscle cells, fibroblasts, keratinocytes and many other cells. In mesenchymal condensations and developing cartilage versican expression precedes aggrecan expression, which is found together with fibulins as in an attempt to organize the early matrix. Neurocan produced by neuronal cells and brevican produced by astrocytes (with the shortest core protein in this family, occurs also in a glycosylphosphatidylinositol -form) are mainly found in the nervous system and participate in glial scar formation and central nervous system repair (Fawcett and Asher 1999).

SLRPs decorin, fibromodulin and lumican reduce collagen fibre thickness of both type I and II collagens. Fibromodulin may also catalyze lateral growth of type I collagen, whereas perlecan with attached chondroitin sulphate can promote fibrillogenesis of type II collagen.

Ground substance also contains non-core protein bound and non-sulphated sixth GAG known as hyaluronan (Gr. Hyalos = glass) composed of D-glucuronate β(1–3) N-acetyl-D-glucosamine β(1–4) backbone and found in e.g. synovial fluid, articular cartilage, vitrous fluid of the eye ball. It can be 25–25,000 disaccharide units long and imparts high viscosity to hyaluronan containing body fluids. Due to its water binding ability a hyaluronan domain occupies some 1,000-fold the volume of its dry state.

GAG-derivatized chitosan membranes increased MSC growth rate about fivefold compared to tissue culture plastic or chitosan alone, but in a GAG-type and concentration-dependent manner. Effects of heparin, heparin sulphate, dermatan sulphate and chondroitin-6-sulphate were fibronectin-dependent, but those of hyaluronan and chondroitin-4-sulphate were fibronectin-independent (Uygun et al. 2009).

2.5 Integrin and Non-Integrin Matrix Receptors

Integrins are 24 different non-covalently coupled heterodimeric cellular receptors composed of 16 α and 8 β chains, which form an important link in the integration of cellular actin cytoskeleton with the cellular surrounding, including the ECM. They do not only bind cells to matrix, but also allow exploration, migration and outside-in and inside-out signalling, which acts in concert with soluble and cell-cell signals in the regulation of cell behaviour. Discoidin domain receptors, Lutheran Lu/B-CAM complex and α/β-dystroglycan complex form important collagen, laminin and/or other ligand binding matrix receptors. Lately lectins, glycan binding receptors, in particular extracellular C-, R- and I-type lectins and galectins have been shown to play roles in cell-matrix adhesion and signalling, including MSC recruitment, bone marrow stem cell niche and adult collagen remodelling.

2.5.1 Integrin-Type Matrix Receptors

Integrins form the major class of cellular receptors for ECM ligands, so much so that the other matrix receptors are often summed up as non-integrin receptors. Integrin receptors are heterodimers, which are composed of one of the 16 α and 8 β chains, which all cross the cell membrane only once. They combine in a non-covalent fashion along currently known combinatorial rules to altogether 24 different integrin receptors (Fig. 2.1). All integrin receptors are able to bind to at least two ligands, which leads to overlap and redundancy and cover many important components of the ECM (Table 2.4). Alternative processing of α and β chains confers further diversity to the integrin receptor system.

The α-chain of the integrin receptor largely determines its ligand binding specificity, whereas the β-chain mainly participates in the assembly of integrins to specialized clusters known as focal adhesions, which mediate external physical stress from extracellular collagen, fibronectin, laminin and other matrix ligands to cellular actin cytoskeleton, i.e. integrate the cell to its matrix. Integrins are often grouped to subfamilies based on their ligand binding specificity, evolutionary relationships and topological restrictions (e.g. leukocyte integrins).

The binding force of one individual integrin-matrix ligand pair is minor compared to other more specialized anchoring transmembrane molecules, but the combined avidity of a myriad of integrins can resist considerable mechanical forces. At the

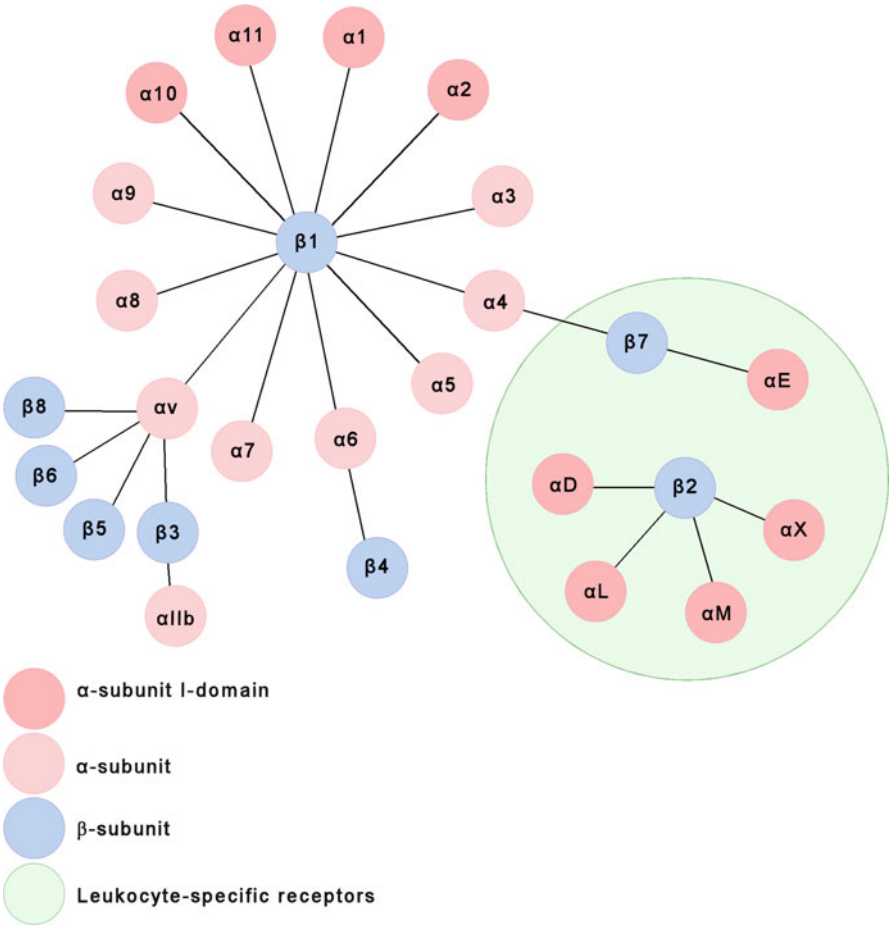


Fig. 2.1 Integrins are heterodimeric receptors composed of one of the 16 known α and 8 β chains. They combine in a non-covalent fashion along currently known combinatorial rules, which are shown in the figure, and which leads to the formation of altogether 24 different integrin receptors

same time this arrangement allows the cell to explore and respond to its extracellular matrix, to bind and to let go, i.e. enable dynamic cellular migration along solid substrates in a process known as haptotaxis. Integrins can form new bonds at the advancing edge of the cells, at the same time when integrin-ligand bonds dissociate at the retracting rear.

Integrins are not passive matrix binders but their expression and binding are actively regulated in a bidirectional inside-out and outside-in signalling, which qualitatively and quantitatively regulates integrin-mediated cell-matrix interactions. Binding to ECM delivers signals regulating migration, proliferation, growth, differentiation and apoptosis, often along the same signal transduction pathways which act in concert with various soluble chemotactic, growth and differentiation factors

Table 2.4 Some non-integrin receptors and their ligands

Receptor	Other names or comments	Ligand
Tyrosine kinase receptors		
DDR1		Collagen I, II, III, IV, V, VI, VIII
DDR2		Collagen I, III, X
Lutheran blood group antigen	B-CAM	Lm-511, -521, -523
Dystroglycan-glycoprotein complex		Lm-111, Lm-211
Lectins		
C-type lectins	Ca^{2+}-dependent	
E-selectins	Endothelial	sLeX, s6SLeX, CD44, CD43
L-selectins	Leukocyte	Collagen XVIII, GlyCAM-1, MadCAM-1, MMR, s6SLeX, versican
P-selectins	Platelet and endothelial	sLeX, s6SLeX, PSGL-1
Macrophage mannose receptor	MMR, CD206	Collagen IV, gelatin, mannose
PLA_2R		Collagen I, IV
R-type lectins	Ricin-like	
Endo180	uPARAP, CD280, contains also C-type lectin domains	Collagens I, II, IV, V, gelatin
I-type lectins	Ig-like domain containing	
Neural cell adhesion molecule	NCAM	Heparin/heparin sulphate, chondroitin sulphate/ neurocan
Galectins	β-Galactoside-binding	β-galactoside
Scavenger receptors		
SR-A1		Collagen I, thrombospondin
SR-A2		Collagen I, thrombospondin
CD36	GPIV, GPIIIb	Collagen I, IV
CD44	Extracellular matrix receptor III	Collagen I, IV, hyaluronan
Annexin A5	Annexin V, anchorin II	Collagen II, X
Glycoprotein VI		Fibril-forming collagens, $(GPO)_{10}$
LAIR-1		Collagen I, III, XVII, $(GPO)_{10}$
LAMR1	34/67 kDa laminin receptor	Lm-111
RHAMM	CD168	Hyaluronan
Glypicans		
Glypican-1		Collagen I

(continued)

Table 2.4 (continued)

Receptor	Other names or comments	Ligand
Syndecans		
Syndecan-1		Collagen I, III, V, fibronectin, Lm-111, Lm-211, thrombospondin, Tn-C, vitronectin
Syndecan-2	Fibroglycan	Collagen I
Syndecan-4	Ryudocan	Collagen I, fibronectin, protein kinase C-α

Heino (2007), Heino and Käpylä (2009), Ekblom et al. (2003), Leitinger and Hohenester (2007), Morais Freitas et al. (2007), Kikkawa et al. (2002), Eckes et al. (1999), Martinez-Pomares et al. (2006), East and Isacke (2002), Wienke et al. (2003), Shimaoka et al. (2002), Bernfield et al. (1992), and Xian et al. (2010)

B-CAM basal cell adhesion molecule, *DDR* discoidin domain receptor, *GlyCAM-1* glycosylation-dependent cell adhesion molecule-1, *GP* glycoprotein, *GPO* glycine-proline-hydroxyproline, *LAIR1* leukocyte-associated immunoglobulin-like receptor-1, *LAMR1* laminin receptor 1, *MAdCAM-1* mucosal addressin cell adhesion molecule, *MR* mannose reseptor, *PLA2R* M-type phospholipase A2 receptor, *PSGL-1* P-selectin glycoprotein ligand-1, *RHAMM* receptor for hyaluronan-mediated motility, *s6SLeX* sialyl 6-sulpho Lewis-X, *SLex* sialyl Lewis-X, *SR* scavenger receptor, *Tn-C* tenancin, *uPARAP* urokinase-type plasminogen activator receptor associated protein

and their receptors. Thus, integrins both bind cell to its surrounding but also help the cell to respond to it. Integrins are not constantly active but their activity is regulated, in part via other integrins.

2.5.2 Non-Integrin Matrix Receptors

Some of the non-integrin receptors are shortly summarized (Table 2.5). Discoidin domain receptors-1 and -2 (DDR1 and DDR2) mediate in its various isoforms cellular non-integrin binding to collagen and are tyrosine kinase receptors, which regulate cell adhesion, proliferation and ECM. DDR1 has been described in cells in brain, skin, colonic mucosa, kidney tubules, lungs and thyroid gland, whereas DDR2 has been found in heart and skeletal muscle, lung, brain and kidney. Cartilage collagen type II stimulates DDR2 and MMP-13 expression in chondrocytes, which parameters are linked to the severity of osteoarthritis (Sunk et al. 2007).

The Lutheran system Lu/B-CAM comprises Lutheran (Lu) and its alternatively spliced form, basal cell adhesion molecule (B-CAM). They are expressed by red blood cells, over-expressed in sickle cells, but also expressed by vascular endothelial cells and epithelial cells. In normal cells they are polarized and in cancer cells they are over-expressed. They bind laminin α_5 containing Lm-511, Lm-521 and Lm-523. Human embryonic stem cells synthesize laminin α_1 and α_5 chains together

Table 2.5 Some non-integrin receptors and their ligands

Receptor	Other names or comments	Ligand
Tyrosine kinase receptors		
DDR1		Collagen I, II, III, IV, V, VI, VIII
DDR2		Collagen I, III, X
Lutheran blood group antigen	B-CAM	Lm-511, -521, -523
Dystroglycan-glycoprotein complex		Lm-111, Lm-211
Lectins		
C-type lectins	Ca^{2+}-dependent	
E-selectins	Endothelial	sLeX, s6SLeX, CD44, CD43
L-selectins	Leukocyte	Collagen XVIII, GlyCAM-1, MadCAM-1, MMR, s6SLeX, versican
P-selectins	Platelet and endothelial	sLeX, s6SLeX, PSGL-1
Macrophage mannose receptor	MMR, CD206	Collagen IV, gelatin, mannose
PLA_2R		Collagen I, IV
R-type lectins	Ricin-like	
Endo180	uPARAP, CD280, contains also C-type lectin domains	Collagens I, II, IV, V, gelatin
I-type lectins	Ig-like domain containing	
Neural cell adhesion molecule	NCAM	Heparin/heparin sulphate, chondroitin sulphate/ neurocan
Galectins	β-galactoside-binding	β-galactoside
Scavenger receptors		
SR-A1		Collagen I, thrombospondin
SR-A2		Collagen I, thrombospondin
CD36	GPIV, GPIIIb	Collagen I, IV
CD44	Extracellular matrix receptor III	Collagen I, IV, hyaluronan
Annexin A5	Annexin V, anchorin II	Collagen II, X
Glycoprotein VI		Fibril-forming collagens, $(GPO)_{10}$
LAIR-1		Collagen I, III, XVII, $(GPO)_{10}$
LAMR1	34/67 kDa laminin receptor	Lm-111
RHAMM	CD168	Hyaluronan
Glypicans		
Glypican-1		Collagen I

(continued)

Table 2.5 (continued)

Receptor	Other names or comments	Ligand
Syndecans		
Syndecan-1		Collagen I, III, V, fibronectin, Lm-111, Lm-211, thrombospondin, Tn-C, vitronectin
Syndecan-2	Fibroglycan	Collagen I
Syndecan-4	Ryudocan	Collagen I, fibronectin, protein kinase C-α

B-CAM basal cell adhesion molecule, *DDR* discoidin domain receptor, *GlyCAM-1* glycosylation-dependent cell adhesion molecule-1, *GP* glycoprotein, *GPO* glycine-proline-hydroxyproline, *LAIR1* leukocyte-associated immunoglobulin-like receptor-1, *LAMR1* laminin receptor 1, *MAdCAM-1* mucosal addressin cell adhesion molecule, *MR* mannose reseptor, *PLA2R* M-type phospholipase A2 receptor, *PSGL-1* P-selectin glycoprotein ligand-1, *RHAMM* receptor for hyaluronan-mediated motility, *s6SLeX* sialyl 6-sulpho Lewis-X, *SLex* sialyl Lewis-X, *SR* scavenger receptor, *Tn-C* tenancin, *uPARAP* urokinase-type plasminogen activator receptor associated protein

with laminin β_1 and γ_1 chains suggesting that Lms-111 and -511 may be important for the their cell-matrix contacts. Correspondingly, functional adhesion experiments suggested that in particular Lutheran blood group antigen and B-CAM together with Int $\alpha_3\beta_1$ play an essential role for their adhesion to Lm-511, whereas Int $\alpha_6\beta_1$ mediated adhesion to Lm-411 (Vuoristo et al. 2009). Such studies are important because one important role for the non-homologous feeder cell layer may be production of ECM, which is necessary for their interactions with stem cells and for stem cell proliferation and maintenance. It might be possible to culture stem cells without feeder cells and to replace stem cell-feeder cell communication by performing stem cell cultures on appropriate matrix substrate.

Alpha-dystroglycan is extracellular molecule, which binds laminin-α_2, agrin and perlecan, whereas the associated transmembranous β-dystroglycan component links the dystroglycan complex intracellularly to dystrophin, which further mediates contact with the actin cytoskeleton. α/β-dystroglycan provides structural integrity and synaptic acetylcholine receptor organization in muscle and other tissues.

Lectins are sugar moiety specific carbohydrate binding non-integrin receptors mediating attachment and aggregation of cells via binding to and cross-linking (at least two sugar binding sites, referred to as carbohydrate-recognition domains) glycoproteins, glycolipids and other glycoconjugates (glycans). Some of them are cell membrane bound. If their glycan ligands locate in the extracellular matrix, they mediate cell-matrix recognition and interactions, but their main task seems to be recognition of various microbial pathogens. Due to their binding specificity, lectin-mediated binding can be regulated by blocking mono- or oligosaccharides, which are useful to study their sugar specificity and have potential as drugs and research tools.

Extracellular lectins include C-type (Ca^{2+}-dependent), R-type (ricin-like), I-type (immunoglobulin domain containing) and galectins (β-galactoside binding),

but new extracellular and intracellular lectin families have been recently described. Selectins (endothelial E-, leukocyte L- and platelet and endothelial P-selectins) belong to C -type lectins, which participate in leukocyte recruitment (tethering and rolling). MSCs seem to lack the conventional P-selectin ligands, P-selectin glycoprotein ligand 1 (PSGL-1) and CD24. They may instead express some novel ligand because P-selectin on endothelial cells induces rolling and tethering of circulating MSCs. Chemokines attract and activate MSCs via chemokine receptors to express the very late activation antigen-4 (VLA-4 = Int $\alpha_4\beta_1$ receptor = CD49d/CD29), which firmly adheres the MSC to the vascular cell adhesion molecule-1 (VCAM-1, CD106) on endothelial cell, enabling transmigration to damaged tissues via diapedesis (Fox et al. 2007; Rüster et al. 2006). Selectins may also play a role in the homing to and maintenance of stem cells in the bone marrow stem cell niche. Endo180 on fibroblasts and macrophages, a member of R (ricin-like)-type lectin, contains fibronectin-like domains, which can mediate binding to e.g. collagens I, II, IV and V. It forms a trimolecular complex with urokinase plasminogen activator (uPA) and its receptor (uPAR), but it is not known if its C-type and R-type lectin domains and glycan recognition sites are important in Endo180-mediated cell-matrix adhesion events. Endo180 is also a collagen internalisation receptor, which together with $\alpha_2\beta_1$ integrin receptors mediate specific binding, cellular uptake and delivery of collagens to intracellular, lysosomal degradation. In addition to its major role in the intracellular collagen degradation, endo180 seems to regulate the other major collagenolytic pathway, namely the extracellular and pericellular MMP-dependent collagen degradation pathway (Messaritou et al. 2009). I (Ig-like domain containing)-type lectins contain many members belonging to the siglec group (sialic acid-binding immunoglobulin superfamily lectins) or other I-type lectins. They have been described on various leukocytes, like macrophages, dendritic cells, B cells, neutrophils, eosinophils etc., but one of the best studied I-lectins is NCAM (neural cell adhesion molecule). NCAM can bind heparin/heparin sulphate containing cell surface and matrix proteins and chondroitin sulphate containing neurocan. It can also indirectly bind to collagen via heparin/heparin sulphate bridges (Angata and Brinkman-van der Linden 2002). Galectins (β-galactoside-binding) are expressed on many immune cells and participate in innate and adaptive responses by modulating T-cell apoptosis, proliferation, adhesion, chemotaxis and synthesis of cytokines and other mediators. They are also expressed on keratinocytes (galectin-7), lung (galectin-8) and adipocytes (galectin-12), where they play roles in skin healing, lung cancer and adipogenic signalling/adipocyte differentiation, respectively. Galectin-1 and -3 have been described to modulate cell-matrix interactions (Rabinovich et al. 2002) and galectin-9 to accelerate TGF-β_3 induced chondrogenic responses (Arikawa et al. 2009).

Some broad-specificity scavenger receptors of class A, B and C may also bind components of extracellular matrix, e.g. CD36 belonging to scavenger receptor type on the surface of platelet can bind it to collagen. Hyaluronan can by bound by hyaluronan cell surface receptor CD44, which has several different isoforms and is found on the surface of chondrocytes and other cells.

2.6 Matrix Modulating Proteinases

Proteinases participate in normal tissue remodelling, but can cause tissue destruction when uncontrolled and excessively active. Proteinases are divided based on their catalytic mechanism to secretory neutral serine and metallo endoproteinases (and amino- and carboxypeptidases) and to mostly intralysosomal acidic cysteine and aspartate endoproteinases. Classification of the proteinases is based on their catalytic mechanisms, which is reflected in the key amino acids necessary for the catalysis. In practice, classification is often based on the use of class specific inhibitors.

2.6.1 Neutral Endoproteinases

Matrix metalloproteinase or MMP family consists of 22 members, subdivided in collagenases, stromelysins and other MMPs (archetypical MMPs); gelatinases; matrilysins; type I and II transmembrane "membrane type MMPs" (MT-MMP), GPI anchored MT-MMPs and secreted MMPs (furin-activatable MMPs) (Table 2.6).

MMPs have so an extended substrate specificity that they can in practice degrade any protein component of the ECM. MMP activity is regulated at the level of gene transcription (cis-regulatory elements and epigenetic mechanisms), translation (mRNA stability, translational efficiency and probably also micro-RNA-mediated), storage/secretion (e.g. pro-MMP8 is stored in neutrophils in the secondary or specific granules), focalization (e.g. MT1-MMP/TIMP-2/MMP-2 complexes), activation (of pro-MMP to MMP) and endogenous inhibitors (tissue inhibitor of metalloproteinases, TIMPs). MMPs are subjected to single nucleotide polymorphism, which can modulate their transcriptional efficiency and disease susceptibility. MMPs have a modular structure, which in archetypical MMPs includes a secretory signal sequence (pre-peptide), an activation peptide (pro-peptide), a catalytic Zn^{2+} containing domain, a hinge region and a hemopexin-like domain. In gelatinases the catalytic domain is flanked by a fibronectin-like domain and the MMP structure may contain a furin activation sequence (furin-activatable MMPs), a transmembrane domain (in transmembrane MT-MMPs), a cytoplasmic tail, a glycophosphatidylinositol (GPI) linker (and a GPI anchor), a cysteine array or an immunoglobulin domain, which regulate various aspects of MMP function, such a substrate specificity, activation and membrane-localization. Classification of MMPs is based on their domain arrangement (Fanjul-Fernández et al. 2010).

When neutral pH prevails in ECM only specialized proteinases, first described in the tadpole tail, collagenases, can degrade across the triple helix at ^{775}Gly–^{776}Ile(Leu), which is the specific initial cleavage site (see below). At normal body temperature ¾- and ¼-degradation fragments formed undergo helix-to-random coil transition to gelatines, which is simply denatured collagen.

MMPs can destroy old or excessive matrix to provide space for cells, such as during vascular invasion, fibroblast or osteoblast migration or chondrocyte alignment. Degradation of cell attachment substrates induces a special form of apoptosis

Table 2.6 Classification of matrix metalloproteinases (MMPs) based on their domain arrangement and their alternative names, molecular weights and their collagenous, non-collagenous and other substrates

Classification	MMP	Alternative names	Mw[a]	Collagen substrates	Non-collagenous substrates	Other substrates
Archetypal MMPs						
Collagenase	1	Collagenase-1	52/41	I, II, III, VII, VIII, X, XI, gelatin	Entactin, fibronectin, laminin, perlecan, proteoglycans, tenascin, vitronectin	α_1-antiprotease, α_1PI, α_2M, casein, C1q, fibrinogen, IL-1α and -β, proMMP-1, -2, pro-TNF-α, SDF-1
	8	Collagenase-2	75/58; 54/42	I, II, III, V, VII, VIII, X, gelatin	Entactin, fibronectin, laminin, proteoglycans, tenascin	ADAMTS-1, α_2M, α_1PI, fibrinogen, Ln-5, proMMP-8, substance P, tissue factor pathway inhibitor
	13	Collagenase-3	60/48	I, II, III, IV,V, VII, IX, X, XIV, XVII, gelatin	Fibronectin, laminin, proteoglycans, tenascin	C1q, fibrinogen, MCP-3, proMMP-9, -13, SDF-1
Stromelysin	3	Stromelysin-1	54/43, 28	IIII, IV, V, VII, IX, X, XI, gelatin	Decorin, elastin, fibronectin, laminin, proteoglycans, tenascin, vitronectin	α_1PI, α_2M, E-cadherin, casein, fibrin, fibrinogen, L-selectin, proHB-EGF, proMMPs, proTNF-α
	10	Stromelysin-2	54/43, 24	I, III, IV, V, IX, X, XI, gelatin	Aggrecan, elastin, fibronectin, integrin, laminin, vitronectin	α_1PI, α_2M, casein, fibrin/fibrinogen, proαdefensin, proMMPs, proTNF-α
Other archetypal MMPs	12	MMP-12, metalloelastase	54/43, 22	I, IV, gelatin	Decorin, elastin, entactin, fibronectin, laminin, proteoglycans, osteonectin, vitronectin	α_1PI, α_2M, casein, E-cadherin, plasminogen, proTNF-α

(continued)

Table 2.6 (continued)

Classification	MMP	Alternative names	Mw[a]	Collagen substrates	Non-collagenous substrates	Other substrates
	19	MMP-19, RASI	57	I, II, IV, gelatin	Aggecan, COMP, entactin, fibronectin, laminin, tenascin	Casein, C1q
	20	MMP-20, enamelysin	54/43	IV, XVIII, gelatin	Aggrecan, amelogenin, COMP, laminin, tenascin,	Casein
	27	MMP-27	75	II, gelatin	Fibronectin	
Gelatinases						
Gelatinases	2	Gelatinase A	72/62	I, II, III, IV, V, VII, X, XI, gelatin	Decorin, fibronectin, elastin, fibrillin, fibulin, laminin, myelin basic, osteonectin, proteoglycans, tenascin, vitronectin	α_1- antiprotease, α_1PI, FGF-R1, IGFBPs, IL-1β, MCP-3, plasminogen, proMMP-9, -13, proTGFβ, proTNF-α, SDF-1, substance P
	9	Gelatinase B	92/82	I, IV, V, VII, X, XI, XIV, XVII, gelatin	Aggrecan, decorin, fibronectin, elastin, laminin, osteonectin, tenascin, vitronectin	α_1-antiprotease, α_2M, α_1PI, C1q, CXCL5, IL-1β, IL-2RA, myelin basic, plasminogen, TGFβ, SDF-1, substance P
Matrilysins						
Matrilysin	7	Matrilysin 1, PUMP-1	28/19	I,II, III, IV, V, IX, X, XI, gelatin	Aggrecan, decorin, elastin, entactin, fibronectin, laminin, tenascin, vitronectin,	α_1PI, E-cadherin, casein, fibrin/fibrinogen, proMMPs, plasminogen, proα-defensin, proTNF-α
	26	Matrilysin 2, endometase	29/19	I, IV, gelatin	Aggrecan, elastin, entactin, fibronectin, laminin, tenascin	α_1PI, E-cadherin, casein, plasminogen, proα-defensin, proMMPs, proTNF-α

Furin-activatable MMPs						
Secreted MMPs	11	Stromelysin-3	55/44	I, IV, gelatin	Elastin, entactin, fibrillin, fibronectin, laminin, proteoglycans, vitronectin	α_1PI, α_2M, factor XII, fibrinogen, IGFBP-1, plasminogen, proTNF-α
	21	MMP-21,	62/49 (human)	Gelatin		
	28	MMP-28, epilysin	59/45			Casein
MT-MMP	14	MT-MMP-1	66/60	I, II, III, IV, gelatin	Aggrecan, fibronectin, entactin, laminin, nidogen, perlecan, vitronectin	α_1PI, α_2M, CD44, factor XII, fibrin, fibrinogen, proMMPs, proTNF-α
	15	MT-MMP-2	76/61	I, gelatin	Aggrecan, entactin, fib ronectin, laminin, nidogen, tenascin, vitronectin	ProMMP-2, proTNF-α, transglutaminase
	16	MT-MMP-3	70/56	I, III, IV, gelatin	Aggrecan, fibronectin, perlecan, vitronectin	Casein, proMMP-2, transglutaminase
	17	MT-MMP-4	71/67	Gelatin	Fibronectin	Fibrin, fibrinogen, α_2M,proTNF-α
	24	MT-MMP-5	73/64	I, gelatin	Fibronectin, laminin, proteoglycans	ProMMP-2
	25	MT-MMP-6	63/58	IV, gelatin	Fibronectin, laminin	ProMMP-2
Type II trans-membrane MMPs	23	MMP-23, CA-MMP	66	Gelatin		

Modified from Visse and Nagase (2003), Murphy and Nagase (2008), and Amalinei et al. (2007)

ADAMTS-1 a desintegrin-like and metalloproteinase with thrombospondin motifs-1, *α_2M* α_2-macroglobulin, *α_1PI* α_1 proteinase inhibitor, *COMP* cartilage oligomeric matrix protein, *Cq1* complement 1q, *CXCL5* chemokine (C-X-C motif) ligand 5, *FGF-R1* fibroblast growth factor -receptor 1, *IGFBPs* insulin-like growth factor binding proteins, *IL-2RA* interleukin-2 receptor alpha, *IL-1β* interleukin-1β, *MCP-3* monocyte chemoattractant protein-3, *MT-MMP* membrane-type MMP, *proHB-EGF* pro heparin binding epidermal growth factor-like growth factor, *PUMP-1* putative metalloproteinase 1, *RASI* rheumatoid arthritis synovium inflamed, *SDF-1* stromal derived factor-1, *TGF-β* transforming growth factor-β, *TNF-α* tumour necrosis-α

[a]Latent/active, kD

in ECM-dependent mesenchymal cells, so called anoikis, but can also release suppressive effects and stimulate cellular proliferation and differentiation. Due to their effects on non-matrix proteins, such as cell surface molecules or heparin-bound matrix deposited factors and activation of latent pro-proteinase zymogens, MMPs can exert various anti-inflammatory and pro-healing effects. The relatively recently recognized fact that MMPs do not only degrade tissues, but also act as signalling scissors, may explain the failure of more or less generalized MMP-inhibitors (Steinmeyer and Konttinen 2006) in the treatment of tissue destructive diseases, such as cancer growth and metastasis.

As has been learnt from tissue engineering constructs, the pore size and interconnectivity have to be appropriate for the cells to migrate into wound healing scaffolds. With natural scaffolding substances such as fibrin and collagen this does not pose much of a problem, because the cells are capable to widen proteolytically too tight pores. In contrast, if the matrix is too sparse, cells sense it and produce more matrix to create extracellular substrate for their integrin and non-integrin matrix receptors adequate for adhesion or directed migration. To at least slightly mimic this natural situation tissue engineering scaffolds are often constructed of bioresorbable (biodegradable) materials, which are hydrolyzed and actively degraded to be replaced by natural matrix. Matrix provides solid substrate along which the cells can migrate to assume their optimal positions in the matrix-cell composite in a process known as haptotaxis or contact guidance, guidance of cellular migration via extracellular matrix ligands instead of soluble chemotactic stimuli. This way extracellular matrix can regulate morphogenesis, would healing and vessel growth as well as pathological cancer cells.

Transmigration and invasion of MSCs requires coordinated action of selectins and glycoproteins, chemokines, integrins and adhesion molecules, cellular cytoskeleton and proteinases and their inhibitors, such as MT1-MMP, MMP-2, TIMP-1, TIMP-2 and TIMP-3 (Ries et al. 2007; Steingen et al. 2008).

Serine proteinase form the largest class of mammalian proteinases, which participate in coagulation, fibrinolysis, complement activation, kininogen metabolism and many other cascades as well as tissue remodelling and destruction. Important enzymes in tissue repair are elastase and cathepsin G in neutrophils and monocytes as well as mast cell tryptase and chymase. Neutrophil elastase is synthesized during the promyelocyte stage, stored in the primary or azurophilic granules and released from triggered neutrophils whereas macrophage metalloelastase is released from activated macrophages. It degrades elastin, but also type III and IV collagens, cartilage proteoglycans, fibronectin and laminin. Elastase can activate pro-MMP-3 (prostromelysin-1) and degrades TIMPs. Cathepsin G is similarly stored and packaged in serglycin matrix in active form and can degrade matrix, activate some pro-MMPs and degrade TIMPs once released. Also plasminogen activators (tissue type and urokinase type), plasmin, plasma kallikrein are considered to take part in degradation of extracellular matrix (Takagi 1996).

Serine proteinases are inhibited α_2 macroglobulin, which utilizes a bait sequence and entrapment, and by specific inhibitors of serine proteinases or serpins. Serpins comprise α_1-antitrypin (α_1-proteinase inhibitor, synthesized mainly in liver, the

main inhibitor of elastase), α_1-antichymotrypsin (acute phase reactant, the main inhibitor of cathepsin G), antithrombin III, α_2-antiplasmin, plasminogen activator inhibitors (PAIs) and C1-inhibitor and protease nexins (e.g. uPA is inhibited by protease nexin-1). High expression of PAI-1 in MSCs seems to associate with a poor migration capacity (Li et al. 2009).

2.6.2 Acidic Endoproteinases

Cathepsins comprise in man 11 members, cathepsins B, C, F, H, K, L, O, S, W, X and Z. Asparate proteinase family has also many members, including cathepsin D and pepsins, which are produced by the chief cells in the stomach and known for their role in digestion. Acidic proteinases become activated by acid and are active in phagolysosomes, in Howship's lacunae below the bone resorbing osteoclasts, in the stomach and extracellularly in acidic pH. They participate in the killing and digestion of microbes, ECM and autologous cellular components (autophagy or autophagocytosis). Apart from pH-dependent regulation of activation, cathepsins are inhibited by endogenous cysteine proteinase inhibitors, cystatins, e.g. the extracellular cystatin C. An acidic cysteine endoproteinase cathepsin K, the major cathepsin of bone resorbing osteoclasts, can cleave across the collagen triple helix at several sites and may play a role also in the extracellular degradation of matrix, not only in the Howship's resorption lacuna but also around loosening joint implants and other acidic locations (Ma et al. 2006). High levels of cathepsin B (a cysteine endoproteinase) and cathepsin D (an aspartate endoproteinase) are associated with a high migration capacity of MSCs (Li et al. 2009).

2.7 Wound Healing

Wound healing occurs in stages, which comprise haemostasis, inflammation, migration, proliferation and differentiation of fibroblasts and angioblasts, re-epithelialization and scar remodelling. Clot, early, intermediate and mature connective tissue matrices interact with the repair cells via integrin and non-integrin receptors so that chemokinetic, mitogenic and differentiation signals and lytic enzymes can be produced in organized waves following one another. Wound healing provides a good model for the study of angiogenesis.

Skin wound healing comprises several stages, which include (1) haemostasis via vasoconstriction, adhesion and aggregation of platelets and activation of the external coagulation cascade to form a temporary blood clot and wound matrix filling the tissue defect and attracting blood leukocytes to the wound, (2) inflammatory protection of the wound site from microbial invasion by neutrophils and monocyte/macrophages, migrating from wound margins along the fibrin- and tenascin-rich temporary scaffold (Badylak 2002; Hodde and Johnson 2007; Ågren and Werthén

2007), and removal of necrotic tissue and blood clot in a proteolytic process, in part orchestrated, neutrophil extracellular traps by lymphocytes via chemokine- and cytokine-mediated processes (Schultz et al. 2005); in addition, these leukocytes produce factors attracting and stimulating, (3) migration of fibroblasts to the lesional site via haptotaxis along fibrin, fibronectin and other components of the temporary wound matrix, fibroblast proliferation, fibroblast-mediated synthesis of subepithelial connective tissue dermal matrix or a more permanent wound matrix, and vascular endothelial cell in-growth and angiogenesis, to form so called granulation tissue, (4) contraction of the open wounds via the action of specialized actin-rich myoepithelial cells, (5) followed by re-epithelialisation by marginal epithelial cells in properly closed (or sutured) wounds and (6) gradual remodelling of the early healing tissue (Clark 1995).

Cells can actively and dynamically assemble and disassemble matrix ligand-integrin receptor attachment areas as platforms to assemble cytoskeletal actin fibres to focal adhesion complexes, which in the subcytolemmal cytoplasm attract and bind many adapter, linker and signalling molecules. Focal adhesions do not only temporarily anchor the cell to matrix, but they are also used for cellular migration via coordinated, directed and extracellularly guided contractions of the contractile cytoskeletal elements. Such phenomena play a role in migration of macrophages, fibroblasts and vascular endothelial cells to wound area and of epithelial cells from the wound margins to the subepithelial healing tissue to cover it again with an intact epithelium.

Dermal fibroblasts contain several integrins, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$. The collagen receptors ($\alpha_1\beta_1$, $\alpha_2\beta_1$) and the fibronectin receptors ($\alpha_5\beta_1$, $\alpha_3\beta_1$ and α_v-integrins) are expressed in the quiescent state and used for adhesion to matrix. It is not completely clear which β-subunits combine with the α_v to form the functional integrin heterodimers *in vivo*. When wounding occurs, quiescent fibroblasts are activated to migrate into the blood clot along collagen fibers or other molecules that cover or associate with the collagen fibers. There is evidence that in early wound-healing, fibroblast migration may be primarily mediated by fibronectin. Migrating fibroblasts express the primary fibronectin receptor $\alpha_5\beta_1$ and $\alpha_3\beta_1$ and in an experimental study migration was blocked with antibodies against $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. Cells at the wound margin down-regulate the expression of the collagen-binding α_1 and α_2 integrins and express α_v integrins that can interact with multiple ligands, including fibronectin, vitronectin, fibrin and fibrinogen. It is not clear how these integrins are used for cell migration *in vivo*. However, there is some evidence that the composition of the ECM is one of the mechanisms that regulate integrin expression during wound-healing (Steffensen et al. 2001; Ågren and Werthén 2007).

Unlike post-natal human skin wounds, which can lead to the development of chronic wounds, foetal skin wounds (<24 weeks gestation) and adult oral mucosal wounds heal rapidly without or only with minimal scarring. Therefore, an oral ulcer, which does not spontaneously heal within 2 weeks, has to be considered as oral cancer until shown otherwise. The reason for this effective healing without scar formation is that foetal and probably oral mucosal skin fibroblasts migrate more swiftly, produce and remodel ECM components faster and transform to wound closing myofibroblasts more rapidly than their adult counterparts (Irwin et al. 1998).

Further, the composition of the provisional and mature wound matrices produced in foetuses and oral mucosa lesions differs slightly from that produced in adult wounds (Bullard et al. 2003).

Composition of the matrix signals to locally involved cells the current state of the wound healing and guides the clearance of necrotic and damaged tissues and synthesis of matrix components via outside-in signalling (Ågren et al. 2000). This ECM-integrin signalling can synergistically utilize signalling pathways, which overlap with those used by soluble growth and differentiation factors and cell-cell signalling. If this well orchestrated cascade of event fails due to obliterating arteriosclerosis or diabetic macro- and microangiopathy, the risk for chronic ulcers increases due to inflammation, fibroblast senescence and uncontrolled proteolysis (Menke et al. 2007). On the other hand, exuberant, nodular and reddish hypertrophic or outright keloid scarring may lead to contractures and aesthetic problems (Robles and Berg 2007).

Endothelial cells contain at least nine different integrin receptors (Martinez-Lemus et al. 2003; Silva et al. 2008). They attach to vascular basement membrane through their laminin, collagen type IV and/or fibronectin binding β_1 integrin receptors, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$, but have alto other specificities (Table 2.4). This process is not passive, because various intracellular endothelial events modulate integrin-ligand binding in inside-out signalling. In addition, pericytes contain $\alpha_7\beta_1$ and $\alpha_8\beta_1$.

Especially cultured and sprouting vascular endothelial cells forming vacuoles and lumen *in vivo* express integrin receptors $\alpha_V\beta_3$ (the classical vitronectin receptor), $\alpha_v\beta_5$ (vitronectin specific receptor) and up-regulate $\alpha_5\beta_1$ (the classical fibronectin RGD receptor), which bind them also to the provisional basement membrane matrix, which in addition to vitronectin may contain fibrinogen, von Willebrand factor, thrombospondin, fibronectin, thrombospondin or thrombin. Also $\alpha_1\beta_1$ and, $\alpha_2\beta_1$ are up-regulated. These integrin receptors as well as VEGFs, angiopoietins, FGF and transforming growth factor-β (TGF-β), are required for endothelial cell activation and angiogenesis because they regulate endothelial cell proliferation, migration, MMP activation and apoptosis (Brooks et al. 1994; Laurens et al. 2009). $\alpha_V\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin inhibitors prevent angiogenesis (Nisato et al. 2003; Laurens et al. 2009). Endothelial cells align themselves actively along the matrix fibres, but also modulate it proteolytically to enable spouting towards the centre of the healing wound (Paweletz and Knierim 1989). In contrast, laminin binding integrin receptor $\alpha_6\beta_1$ may promote endothelial cell differentiation and stabilization (Davis and Senger 2005).

Syndecan, a cell surface heparin sulphate proteoglycan, binds endothelial cells to heparin-binding domains of matrix fibrillar collagen, laminin, fibronectin, vitronectin and thrombospondin. It seems that when syndecan-1 comes into contact, probably via lateral interactions, with integrin receptors $\alpha_V\beta_3$ and $\alpha_v\beta_5$ they are clustered and activated (Beauvais et al. 2009). Various isoforms of the hyaluronan receptor CD44 mediate binding to hyaluronan, fibronectin and collagen and may also stimulate angiogenesis. It is expressed on vascular endothelial cells in granulation tissue.

Keratinocytes contain several integrins, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_9\beta_1$ and $\alpha_v\beta_5$ as well as the hemidesmosomal component $\alpha_6\beta_4$. To enable rapid

migration, keratinocytes dissolve their hemidesmosomal complexes. Keratinocytes can then migrate on or through the provisional blood clot matrix or, in dermal wounds, under the clot in contact with dermal type I collagen-rich matrix, which require different and only partly overlapping set of integrins for cellular adhesion/migration, signalling and focalized proteolysis (Steffensen et al. 2001).

Venous, arterial and neuropathic leg ulcers form an increasingly important health problem leading to pain, complicating infections, amputations, and decreased quality of life. One strategy is to fill the persisting tissue defect with matrix biomaterial to push it through this bottleneck and to support fibroblast and endothelial cell adhesion, guided migration and local synthesis of ECM to fill the defect and to promote healing through the final stage of the natural wound healing process as described above. One biomaterial used for this purpose is Matrigel, a gelatinous basement membrane-like protein substrate composed of laminin and collagen matrix with growth factors deposited in it. Further, such relatively loose matrices exert mechanotranduction by transferring stains from ECM to cells embedded in or on it, which may stimulate matrix synthesis or induce directional sprouting (branching) and angiogenesis. This is an example how integrin and non-integrin matrix receptors modulate cellular behaviour. Another such proteins is amelogenin, an ECM protein that self-assembles into globular micron-size aggregates, which are able to provide provisional matrix for cell attachment and healing of chronic wounds as described above.

2.8 Cartilage Extracellular Matrix and Regeneration

Cartilage is composed of chondrons, which are organized in lines and connected by interterritorial matrix. In addition, cartilage contains endogenous mesenchymal progenitor cells. Superficial (tangential), middle (radial) and deep (hypertrophic) zone are separated by a tidemark from the calcified cartilage matrix lying on bone. Cartilage contains many growth factors, like TGF-β and IGF-1, and chondrocytes released factors like HMGB-1, which together with the integrin and non-integrin mediated matrix contacts regulate the behaviour of the chondrocytes and matrix production and composition. Against earlier dogma, cartilage contains some mesenchymal stromal cells in its superficial layers. Stimulated by success of the autologous chondrocyte transplantation, these features are simulated in bioresorbable natural and synthetic scaffolds in tissue engineering applications.

2.8.1 Cartilage Structure

Cartilage can be divided to hyaline articular cartilage (rich in type II collagen), menisceal and other adapter fibrocartilages (rich in type I collagen), and elastic cartilage (rich in elastin, e.g. in the ear and epiglottis). The basic unit of the cartilage

is chondron, which typically contains several lacunar chondrocytes, formed by division from the same mother cell (isogenous), each surrounded by its pericellular matrix and with the whole unit being surrounded by territorial matrix. Chondrons are often organized in radial lines and separated from each other by interterritorial matrix, which has a composition different from the intraterritorial matrix in the chondrons (Poole 1992). Hyaline articular cartilage on the surface of long bones in synovial joints is, due to its apparent role in osteoarthritis, of prime interest.

Hyaline articular cartilage ECM contains a fibrous network of type II collagen embedded in PG and glycoprotein-rich hydrophilic ground substance. Hydrophilic heparan sulphate- and keratan sulphate-rich proteoglycans assemble via link proteins to huge macromolecular complexes organized around a HA core (Allemann et al. 2001). Pericellular matrix contains a collagen type VI-rich basket for chondrocytes, but also contains proteoglycans, fibronectin and structure enforcing type II and IX collagen. Matrix proteoglycans decorin, biglycan and fibromodulin form deposits of various soluble factors, such as TGF-β_1, IGFs and BMPs, which upon proteolytic release couple matrix degradation with neosynthesis of cartilage matrix (van der Kraan et al. 2002; Sekiya et al. 2001). Cellular adhesion via integrin ligation regulates the activity of some growth factor receptors. Apart from paracrine factors, chondrogenesis by juvenile chondrocytes seems to acquire autocrine morphogens, which are inhibited by serum-derived growth factors. Articular chondrocytes may lose their capacity to proliferate and maintain cartilage homeostasis at the onset of puberty (Solchaga et al. 2001).

Non-calcified articular cartilage is subdivided into three zones: superficial or tangential, middle, intermediate or radial, and deep or hypertrophic zones. Cartilage type II collagen forms about 60% of the dry weight of the articular cartilage. Small 10 nm fibres are distributed in all three zones. In addition to that, the superficial zone, subjected to high shear forces, is enforced by 35 nm fibres oriented parallel to the cartilage surface; the middle zone contains 70–100 nm randomly organized fibres; and the deep zone contains ~140 nm radially organized fibres. Thickness and organization of type II collagen fibres, as well as their intermolecular linkage, are organized by trace amounts of types I, VI, X, and XI collagens (Naumann et al. 2002). Cartilage tissue is anisotrophic.

The superficial zone of hyaline articular cartilage contains tangentially orientated flattened chondrocytes aligned along the tightly packed type II collagen fibre layers, providing resistance to shear forces of the articular gliding pair and protection to the deeper layer of the cartilage. Middle zone is composed of obliquely oriented collagen fibre meshes resisting compressive forces and serving as a transition zone between the superficial and deeper layers subjected to compressive forces. Tidemark is the interface between the deep hypertrophic cartilage zone and the underlying calcified cartilage, which tidemark can in osteoarthritic cartilage be multiplied (Alford and Cole 2005) and contains deposits of high mobility group box-1 (HMGB-1; Heinola et al. 2010). HMGB-1 is a non-histone, DNA-binding protein, which regulates gene transcription, but released into the extracellular space from necrotic, activated or perhaps even apoptotic hypertrophic chondrocytes acts as an endogenous alarmin and a master cytokine. It can also recruit osteoblasts, vascular

endothelial cells and monocyte/macrophages as well as stimulate transdifferentiation of MSCs to osteoblasts. Its release into extracellular matrix and deposition in the tidemark are dependent on the state of the OA changes (Heinola et al. 2010). Such a delicate tissue and macromolecular organization posses a real challenge to tissue engineering attempts. Against earlier dogma, apart from chondrocytes also mesenchymal progenitor cells have been identified in healthy human articular cartilage (Alsalameh et al. 2004).

Collagen facilitates integrin- and non-integrin-mediated adhesion to matrix, which regulates cellular attachment, spreading, migration, differentiation and survival of the chondrocytes (Kleinman et al. 1981). To avoid a special form of apoptosis, called anoikis, chondrocytes need to attach to collagen fibres using their $\alpha_1\beta_1$ integrin receptors (van der Kraan et al. 2002). By their counterforce against the swelling pressure exerted by the matrix embedding hydrophilic ground substance hydrogels collagens determine the viscoelastic and compressive properties of the hyaline articular cartilage. Protein core attached keratin and chondroitin sulphate side-chain glycosaminoglycans of aggrecan molecules are negatively charged, displaying high electrostatic repulsion and hydrophilicity.

2.8.2 Culture on Different Scaffolds

The aim of cartilage tissue engineering scaffolds is manifold. Optimally they allow cell seeding, they mimic the 3D environment of the ECM, provide preferably temporary and resorbable structural support, an increased surface area-to-volume ratio promoting cellular adhesion, migration and differentiation and integrate with host tissues (Capito and Spector 2003; van Osch et al. 2009).

Cartilage regeneration requires chondrocytes or MSCs able to differentiate to chondrocytes in numbers high enough to enable production and maintenance of hyaline articular cartilage under the demanding physicochemical articular circumstances (Getgood et al. 2009). ECM/scaffold-cell contacts in solid or gel-like scaffolds composed of or containing agarose, alginate, carbon nanotubes, chitosan, chondroitin sulphate, collagens, elastin-like polypeptides, fibrin, gelatine, hyaluronan, polycaprolactone, polylactic acid (PLA), polyglycolic acid (PGA) and polylactide-co-glycolide copolymer (PLGA) are used to support such a development. All these scaffolds can be coated with adsorbed proteins or immobilized functional groups. We know of no studies that have evaluated chondrocyte function on type II collagen sponges spiked with cross-linked CS and hyaluronate. On the other hand, before mass production and clinical use of any such scaffolds, a balance must be reached between bioactivity/-compatibility and production costs.

Apart from its chemical composition, also the shear stress forces, loading and microarchitecture of the cartilage play a role in mechanotransduction and guidance. Optimal pore size and interconnectivity may at the cellular level be rather similar in different species. Devitalized, porous chicken knee 3D scaffold has been reported to form a good framework for bovine neocartilage formation (Warden et al. 2004).

Over 95% porous artificial collagen sponges, produced from approximately 0.5 wt. % collagen solutions, can by lyophilized and physically or chemically crosslinked to obtain 120–200 μm pore size and permissive interconnectivity (Kato et al. 1995). Crosslinking and porosity prevented formation of amorphous hydrogels to which the cells can not migrate (Kato et al. 1995). It is possible to follow expression of chondrocyte genes of aggrecan core protein and collagen type II and accumulation of cartilage matrix in such porous 3D sponges (Glowacki and Mizuno 2007; Lu et al. 2001; Mizuno and Glowacki 1996; Yates et al. 2005). Collagen scaffold-autologous chondrocyte tissue engineering constructs improve histological repair over controls of articular cartilage defects in rabbit, sheep and other experimental models (Lu et al. 2001). MSCs seeded in a collagen gel developed first at 12 weeks hyaline-like repair tissue and subchondral bone, but 12 weeks later articular cartilage was thin and incompletely integrated with host tissues (Wakitani et al. 1994). In contrast, in 2D monolayer cultured chondrocytes soon dedifferentiate to fibroblast-like cells, which produce tough fibrous-type interstitial type I collagen-rich matrix rather than hydrated elastic cartilage matrix.

For cell culture foetal bovine serum, rich in various but poorly defined as to its growth factor content, is used to support cellular proliferation and growth *in vitro*. Due to its heterologous nature, this may cause adverse inflammatory or immunological reactions. This has stimulated attempts to develop well defined synthetic cell culture media, which may contain critical growth factors like transforming growth factor-β. Feeder cells could perhaps be used with tissue engineering scaffolds containing appropriate ECM molecules. Reprogramming of already differentiated cells using gene transduction offer a third option to produce and guide stem cells along the desired cell lineage.

When the distance of a cell from the surface of a 3D scaffold increases, diffusion of nutrients and oxygen to cells in the implant centre is impaired. Bioreactors and solvent flow have been used to extend this distance. Perfusion also exposes cells to shear stress and hydrostatic pressure dependent on the flow rates and other conditions applied. Under such dynamic culture conditions both adherent stromal and non-adherent haematopoietic cells are more evenly distributed to the 3D implants and usually display improved viability and matrix deposition compared to static culture conditions (Shanbhag et al. 2005; Nichols et al. 2009).

Scaffolds seeded with genetically engineered chondrocytes, transduced with bone morphogenic protein-2, produced at 6 months in a rabbit model hyaline-like repair tissue biochemically and biomechanically similar to native tissue, whereas empty collagen sponges was compressed and adhered to the underlying structures (Wakitani et al. 1994).

Chitosan is a bi-copolymer of glucosamine and N-acetylglucosamine. Its degradation products are non-toxic and can be used in the synthesis of articular cartilage (Guo et al. 2006). Chitosan is cationic and, due to its high charge density in acidic solutions, forms water-insoluble complexes with a variety of polyanionic substances, including some growth factors. Chitosan/glycerol copolymer hydrogel (BST Cargel, Biosyntech, Quebec, Canada) mixed with blood and injected into a chondral defect following microfracture provided in a rabbit model better results

than microfracture alone and results from an ongoing human trial are awaited (Hoemann et al. 2007).

Chondroitin sulphate can be used to enhance growth factor binding capacity/proliferation and biocompatibility/matrix deposition of collagen-based scaffolds (Veilleux and Spector 2005). The major GAG of native cartilage tissue is chondroitin sulphate as clarified above.

Chondrocytes growing in a fibrin scaffold produced IGF-1 and produce type II collagen rich matrix (Fortier et al. 2002).

Hyaluronan is a hydrophilic macromolecular component of the ECM. Chondrocytes use various isoforms of hyaluronan receptor CD44 to attach and read hyaluronan. This stimulates chondrogenesis of MSCs by itself and matrix formation in collagen scaffolds is enhanced by addition of small amounts of hyaluronan. Hyaluronan can be cross-linked by esterification, glutaraldehyde or otherwise to produce hylan implants for cartilage repair, but such modification of the natural molecule impairs its biocompatibility and can lead to formation of degradation products that can cause chondrolysis (Knudson et al. 2000).

We believe that natural 3D scaffold materials, such as collagen, chitosan, chondroitin sulphate and hyaluronan, have an edge over synthetic products because they are better provided with cues involved with cellular adhesion and signalling at the same time when resorption can be designed to lead to reciprocal release of matrix deposited growth factors, to couple scaffold degradation with matrix formation.

Synthetic scaffolds produced of PLA, PGA or PLGA are easy to mould and produce and it is possible to control the speed of their biodegradation (Capito and Spector 2003). Carbon nanotube composites support chondrocyte proliferation and deposition of ECM (Khang et al. 2008). Similarly, collagen fibre-mimicking electrospun polycaprolactone nanofibre scaffolds support MSC-chondrocyte differentiation (Li et al. 2005b).

The future of cartilage repair and restoration is promising and the role of ECM in this process is pivotal.

2.9 Bone

Bones (skeleton) provide support and define the shape and form of the body. Relatively rigid bones together with synovial, fibrous, cartilagineous (and bony) articulations and mechanically well placed insertions of ligaments, tendons and joint capsules make locomotion and guidance of it possible. Some parts of the skeleton protect internal organs and bones themselves contain bone marrow with hematopoietic and mesenchymal stromal cells and most of the calcium and phosphate mineral deposits of the body. Bone represents one of the most dynamic remodelling tissues of the body so that osteoclasts, some mononuclear cells and osteoblasts form temporary bone basic multicellular units (BMU), which undergo activation-resorption-formation (ARF) cycles in bone remodelling compartments. Bone lining cells and lacunar osteocytes cover the outer and inner bone surface and the whole

bone is surrounded by periosteum containing a protective outer fibrous and an inner cambian cell layer containing progenitors activated upon fracture and fracture healing. MSCs seeded on scaffolds can be induced to bone to replace various types of bone defects.

According to mode of mineralization, bone is divided into intramembranous bone (direct mineralization, e.g. the flat bones of the skull) and enchondral bone (formation of a cartilage model precedes mineralisation of the matrix, e.g. the long bones of the extremities). Bone is further divided into compact cortical and spongy trabecular bone. The basic unit of cortical bone is an osteon or a Haversian system, which is 0.2 mm in diameter and typically several millimetres long. It is composed of concentric bone lamellae, the interfaces of which are marked by lacunar osteocytes organized into cellular rings interconnected with bone canaliculi around a central Haversian canal, which houses blood vessels.

In addition to bone matrix entrapped osteocytes, bone contains bone matrix forming osteoblasts on the surface of the osteoid (newly forming bone), bone resorbing multinuclear osteoclasts in resorption lacunae and lining cells covering the surface of resting bone (resting osteoblasts). Bone is typically covered by periosteum composed of an outer protective fibrous layer and an inner cambium layer containing osteoblast progenitors.

Bone cells form the basic multicellular unit (BMU), which is a temporary cellular structure composed of a few osteoclasts, which form the cutting cone resorbing bone, followed by a closing cone, in which new bone is formed by osteoblasts on the wall of the cavity formed by the osteoclasts. The transition zone between the cutting and closing cone is covered by relatively poorly defined mononuclear cells, which seem to clean and prepare the bone surface after osteoclast-mediated bone resorption for the attachment and bone synthesis work of the osteoblasts. Osteoclasts resorb the bone only a week or two, but the subsequent *de novo* bone synthesis in the closing cone takes a few (6–7) months.

BMU nicely reflects different phases in the bone remodelling cycle, referred to as activation-reversal-formation (ARF) cycle. Activation leads to the fusion of osteoclast progenitors belonging to the monocyte/macrophage cell linage to multinuclear osteoclasts, which during the resorption phase lyse bone tissue. This resorption phase is via release of matrix bound molecules, S1P/S1PRs and EphB4/ephrinB2 coupled to osteoblast-mediated bone formation. In adult human skeleton the loss of bone and the subsequent formation of bone balance each other so that a *status quo* remains. It is not quite known what activates the ARF cycle, but microfractures of old and strained bone, leading to osteocyte death and release of osteocyte factors, may initiate the cycle and in part direct the BMU-mediated ARF activity towards microfractures.

The special feature of bone tissue is that it is a composite consisting of type I collagen-rich organic matrix (for other bone collagens, see Table 2.1) in which impure 50 nm long, 28 nm wide and 2 nm thick nano-size hydroxyapatite crystals (Cui et al. 2007) have precipitated as bone mineral during primary (rapid mineralization of newly formed osteoid seams) and secondary (slowly increasing mineralization of already formed bone) mineralization (Rho et al. 1998; Veit et al. 2006).

Bone is an ingenious natural material, a composite which consists of solid cortical and porous trabecular, polymeric and ceramic, and lamellar (a few microns thick) and woven (fibrous) phases (Canty and Kadler 2005) organized from nano- to macroscale. Toughness and visco-elasticity of bone depend on its collagen matrix (Young's modulus 1–2 GPa, an ultimate tensile strength 50–1,000 MPa), which also defines the shape and volume of a bone, whereas the hardness and mechanical strength (Young's modulus 130 GPa, an ultimate tensile strength 100 MPa; Park and Lakes 1992) but also brittleness are attributed to hydroxyapatite composed of calcium, phosphorus, hydroxyl ions containing trace amounts of fluoride, sodium, magnesium and other ions (Gray et al. 1995). Non-collagenous proteins (NCP) of bone regulate impregnation of hydroxyapatite crystals in the organic bone collagen matrix framework. High energy fractures cause fractures even in young patients, whereas pathological, low energy fractures occur more commonly in elderly osteoporotic individuals.

In bone growth and fracture healing bone can form *de novo* from MSCs as a result of osteoinduction. This involves several MSC β_1 integrin receptors, including collagen receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$, fibronectin receptor $\alpha_5\beta_1$ and laminin receptor $\alpha_6\beta_1$, but also multi-specific $\alpha_v\beta_3$ and $\alpha_V\beta_5$ receptors, which bind e.g. vitronectin and osteopontin (Gronthos et al. 2001). Osteoconduction or bone in-growth, as tissue regeneration in general, can be guided by haptotaxis and physical barriers. Migration of MSCs through tissues can be aided by $\alpha_v\beta_3$, which can bind and, focalize, MMP-2 to the leading edge of the migrating cells (Karadag and Fisher 2006). A well fixed implant in bone seems at the light microscopic level to be in direct contact with the surrounding bone although high resolution electronmicroscopic images disclose a thin fibrous interface tissue even in these so called osteointegrated implants (Hutmacher et al. 1998).

According to the law of Wolff, the orientation, density, crosslinking and mineralization of the bone trabeculae adjust according to the local mechanical needs, in which mechanotransduction process interacts between bone cells (Table 2.4) and bone collagen (Table 2.1) and NCPs play a role at the sensing and transducing interface (Taylor 2007).

Bone tissue engineering aims to repair/regenerate bone defects, such as congenital, iatrogenic and non-unions. To avoid immunological rejection, usually autologous osteoblasts or MSCs are seeded to biodegradable (more rarely to biostable) but temporarily supporting and void filling porous scaffolds, which hybrids are referred to as tissue engineering constructs (TEC) or products (TEP). Scaffold is in a few milliseconds dynamically coated by soluble plasma/interstitial proteins, which upon maturation is more or less replaced first by provisional and finally by more mature ECM synthesized by the inmigrating scaffold colonizing cells. The natural bone milieu, with its embedded growth and differentiation factors, supports this process. The purpose designed scaffold should retain its strength from a few weeks to several months depending on the purpose of its use. Gradually during a year or two the bioresorbable scaffold should be hydrolytically degraded to water and carbon dioxide without causing a foreign body reaction. This loss of external implant support protects against stress shielding, bone weakening and pathological peri-implant fractures and allows bone formation and remodelling, which lead to the replacement of the artificial construct with natural living bone (Hutmacher 2000).

Both natural and synthetic materials have been tried in bone tissue engineering, including polymers, such as gelatine, agar, fibrin, collagen, brushite and demineralised bone matrix; synthetic bioresorbable polymers, such as polyglycolic-lactic acid (PGLA), poly-L,D-lactic acid (PLDLA) or polycaprolactone (PCL); and porous ceramics, such as bioglass, hydroxyapatite composites or various other calcium phosphate compounds, or naturally occurring ceramics, such as coral. Composites of natural and synthetic materials, such as collagen–PLA-hydroxyapatite and chitosan–hydroxyapatite composites, have been studied. Just like in chondrogenesis, also the architecture of the scaffold matters and both human MSCs (from various sources) and human embryonic stem cells form bone better in natural 3D than on artefactual 2D scaffolds.

Fracture repair is a sequential process that requires a coordinated action of cells, signalling molecules and extracellular matrices. The sequence of events is initiated by the blood clot (provisional healing fibrin mesh scaffold) and ends up with organized, mature and remodelling lamellar bone. Intermediate steps can include formation of granulation tissue, cartilage (in enchondral bone formation) and woven bone.

2.10 Extracellular Matrix in Heart Regeneration

Ischemic heart disease is a common cause for angina pectoris, myocardial infarction and chronic heart disease, which in cases refractory to medicinal treatment cause a great clinical problem. Endogenous repair ability of necrotic heart muscle tissue or fibrous heart valvular tissue is poor. Therefore, attempts have been made to treat these patients with cell therapies, guided tissue regeneration and tissue engineering constructs. Many different cell types (MSC, cardiac stem cell, endothelial progenitor cell, embryonic stem cells, multipotent adult stem cells, skeletal myoblasts or hematopoietic stem cell) and processes (transmigration/extravasation, migration and engraftment, angiogenesis, proliferation, apoptosis, stem cell produced paracrine factors, cardiomyogenesis, arrhythmias) have to be mastered in clinical trials before these therapies can be more widely applied. Interestingly, these cell based therapies do not seem to act so much as cell replacement therapies as to act via complicated orchestration of the repair and regeneration.

2.10.1 Integrins in the Cell Cycle Withdrawal of Cardiomyocytes

Integrins have been studied in the developing heart. Int β_1A chain was present in the proliferating cardiomyocytes but decreased with 30% after birth. Integrin β_1D was found a little later, at the foetal day 18, increased 2 days after birth and remained then constant, which resulted in 1:1 ratio of these integrin chains in the adult heart.

Adhesion to matrix via these β_1 integrins may drive cardiomyocyte proliferation, but this effect is lost in the neonatal heart and upon β_1A to β_1D shift. The adult ß$_1$D form in particular plays a role in anchorage and transmission of mechanical load to heart tissue during heart beats (Sun et al. 2003). After birth β_1 integrin binding partners, the α chains of the heterodimeric laminin integrin receptors, are α_3 and α_6, which are transiently increased at neonatal day 2, while three different isoforms of α_7 chains, part of the LM-111 receptor, increased gradually to the adult stage. Fibronectin receptor $\alpha_5\beta_1$ did not change during foetal-to-neonatal transition. Because cardiomyocyte proliferation ceases upon terminal differentiation shortly after birth, it is assumed that the above mentioned integrin changes initiate cell cycle withdrawal, inhibit S-phase entry and block the cells in G1 phase (Maitra et al. 2000).

2.10.2 Heart Diseases

Angina pectoris refers to ischemia of the heart muscle, usually caused by stenosis of one or more of the coronary arteries impairing local blood delivery critically upon strenuous physical or mental activities, but it can also be caused by anaemia (universally diminished oxygen delivery) or hypertrophic heart diseases and/or rapid arrhythmias (increased oxygen consumption). Myocardial infarction (MI) upon rupture of the atherosclerotic plaque and subsequent intra-arterial blood clot (thrombosis) formation leads to ischemia, then injury and finally necrosis of cardiac tissue. One of the important clinical consequences of atherosclerotic heart diseases is chronic heart failure leading to dyspnoea, fatigue, swelling, increased jugular vein pressure etc.

MI triggers rapidly a process of repair to maintain the structural integrity of the heart. Signal transduction involves cell-cell signalling, but also soluble factors and ECM-cell signalling. Inflammatory leukocytes invade the injured area, angiogenesis ensues, and fibroblasts start to replicate. This early inflammatory phase of healing results in granulation tissue. In large transmural Q-wave MIs the entire heart may be engaged in the repair, which leads to diffuse and widespread fibrosis and remodelling also at sites remote from the actual infarct area. Postinfarction healing is almost complete 6–8 weeks following MI, but the infarct scar, which was once considered to be relatively inert, is quite a dynamic remodelling tissue.

During the inflammation phase leukocyte produce pro-inflammatory cytokines, such as TNF-α and IL-1ß, which up-regulate local synthesis, secretion and activation of matrix metalloproteinases (e.g. MMP-2 and -9). In this early phase, these cytokines play a protective and coordinating role so that anti–IL-1ß impairs repair (Hwang et al. 2001). Similarly, glucocorticosteroids leads to poor healing. MSCs can stimulate fibroblasts to produce MMP-2, MMP-9 and MT1-MMP, which reduces post-MI fibrosis (Mias et al. 2009). Still remaining cardiomyocytes hypertrophy.

Necrotic tissue is gradually phagocytosed and replaced with a collagen-rich scar (Deten et al. 2002). Diffuse fibrosis develops to enforce the heart and cells are via

integrin and non-integrin matrix receptors integrated to this fibrous scaffold. Particularly $ß_1$ integrins seem to be associated with anchoring of the myocytes to matrix. Contractile myofibroblasts proliferate and angiogenesis ensue and, to a limited extent, progenitor cells proliferate (Deten et al. 2001; Sun et al. 2003).

MMPs are present in healthy myocardial cells and interstitium, but only in low concentrations and mostly as latent zymogens. They are readily activated within minutes of ischemia by free radicals and proteinases, such as plasmin and other MMPs. The ratio between MMPs and tissue inhibitors of metalloproteinases (TIMPs) shifts in favour for MMPs (Creemers et al. 2001).

2.10.3 Cell Therapies

Cell therapies have emerged as a potential new mode of treatment in a variety of cardiac diseases, including acute MI, refractory angina pectoris and chronic heart failure (Menasche 2009). Attention has been drawn to selection of appropriate cells, their delivery via myocardial implantation *vs*. intracoronary or systemic infusion, and creation of a specific extracellular matrix niche to promote engraftment, survival and function of the transplanted cells. Transplantable cells can be embedded in fibrin or peptide nanofibres, which seem to enhance graft retention due to higher viscosity of the injectable graft and improved cellular viability, to create a 3D environment and to improve cellular cohesion, cell-to-cell and cell-to-matrix contacts and signalling. Biomaterial "shell" may protect cells from inflammatory and immune damage inflicted by host neutrophils, monocyte/macrophages, lymphocytes and other cells. ECM of transplanted cells could be supplied with agents promoting homing, migration, engraftment, proliferation and differentiation of these cells (Fig. 2.2). Repopulation of the damaged zone with contractile or regulatory cells and beneficial modulation of matrix may help to normalize the hemodynamic load on the surviving cardiomyocytes and the potentially deleterious consequences of ventricular remodelling (Penn and Mangi 2008).

Injury and/or repair triggered induction of stem cell homing factors in myocardial tissue lead to homing of bone marrow derived and cardiac stem cells to the injured myocardium. Re-expression of laminin-1 (Lm-111) in the adult human heart revokes in part mechanisms, which were operative during organogenesis but are now engaged in heart repair. Lm-111 in the ischemic heart may help to create a niche permissive for epithelial-mesenchymal transition in the adult heart, whereas laminin-2 (Lm-211) seems to be essential for the maintenance of already existing cardiac muscle cells. Binding of extracellular heart matrix ligands by integrins results in signal transduction across the plasma membrane that regulate cell shape, migration, growth, and survival, a process termed outside-in signalling.

Adhesion molecules and integrins (Wu et al. 2007; Ip et al. 2007) play a role in the mobilization, engraftment and migration of stem cells through injured myocardial tissue and in the modulation of their connective tissue microenvironment

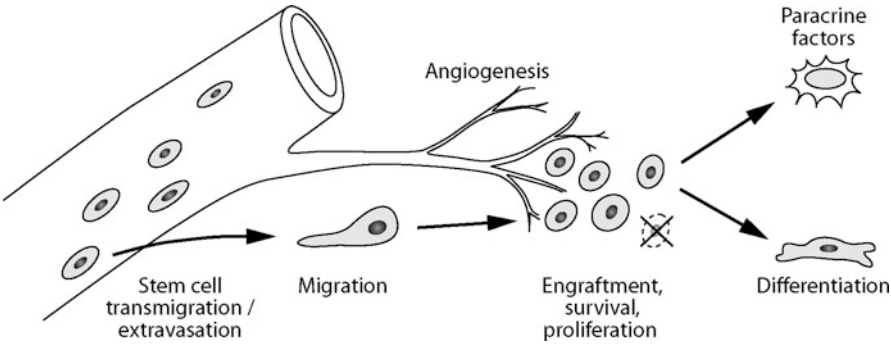


Fig. 2.2 As a result of tissue injury, MSCs have been mobilized from stem cell niches or injected into the circulation. At the site of the injury they transmigrate (extravasate) through the blood vessel wall to tissues or simply flow there with blood and become embedded in the blood clot. They migrate in the interstitial tissues towards the injured area and engraft, but in spite of a growing distance from the source of oxygen and nutrients, they have to maintain their vitality to proliferate. Locally activated or injected stem and progenitor cells may participate in the process. The mainstream idea is nowadays that the stem cells produce locally factors and effects, which promote tissue repair and healing via paracrine mode of actions on a mix of some local resident or immigrant cells, but some of them may terminally differentiate to replace lost resident cells, according to the older view (Modified from Penn and Mangi 2008)

(Xiang et al. 2005; Shimazaki et al. 2008; Tamaoki et al. 2005). Several proteinases, which are potential targets for gene based modulation prior to stem cell transplantation, participate in these processes (Xiang et al. 2005). Inhibitors of MMP activation, such as plasmínogen activator inhibitor-1 (PAI-1), affect leukocyte infiltration and remodelling of the left ventricle (Askari et al. 2003; Xiang et al. 2004). Up-regulation of PAI-1 decreased tissue-type plasmin (tPA) activity, which consequently diminished leukocyte infiltration and tissue degradation and decreased left ventricular dilation (Askari et al. 2003). Down-regulation of PAI-1 associated with increased tPA activity at the time of acute myocardial infarction increased engraftment of exogenously delivered $CD34^+$ cells in the infarct zone due to enhanced vitronectin-dependent transendothelial migration from the blood stream (Xiang et al. 2004). This increase in stem cell engraftment after MI following PAI-1 inhibition was recently shown to associate with a decrease in cardiac myocyte apoptosis and an improvement of heart function (Xiang et al. 2005).

Many attempts have been done to produce scaffolds that directly via their composition or indirectly via matrix remodelling enable myocardial grafts (Xiang et al. 2004, 2005). Particular interest has been paid to three factors: tenascin-C (Tamaoki et al. 2005), relaxin (Perna et al. 2005) and periostin (Litvin et al. 2006).

Tenascin-C is a provisional extracellular matrix molecule that is expressed during wound healing in various tissues, including myocardium following acute MI (Imanaka-Yoshida et al. 2001). Tenascin-C, up-regulated by angiotensin II (Nishioka et al. 2007), seems to be profibrotic. Although it is crucial for normal healing, its down-regulation associates with improved long-term outcome. Tenascin-C accelerates

fibroblast migration and α-smooth muscle actin expression (myofibroblast formation). Increased serum tenascin-C indicates pathologically increased remodelling (Sato et al. 2006).

Relaxin is a hormone belonging to the relaxin superfamily, which also includes insulin-like peptides. Relaxin liberates NO and causes vasodilatation. Administration of relaxin decreased myonecrosis, cardiac myocyte apoptosis and leukocyte infiltration into the injured myocardium after an experimental MI (Perna et al. 2005). Chronic over-expression of relaxin in C2C12/RLX myoblasts increased local MMP-2, VEGF, vascular density and cardiac function compared to untreated and control animals treated with C2C12/GFP (green fluorescent protein) myoblasts alone (Bani et al. 2009).

Periostin is a secreted ECM protein, which regulates left ventricular remodelling, stem cell engraftment and differentiation in multiple heart diseases (Katsuragi et al. 2004). Periostin is found in cardiac fibroblasts and up-regulated in the ECM of the heart following MI. When periostin was injected into the infarct zone following acute MI, it caused adult cardiomyocytes to re-enter the cell cycle. This was associated with the activation of α_v, β_1, β_3 and β_5 integrins and signalling via phosphatidylinositol-3 (PI3K) pathway. This was followed by improved ventricular remodelling and cardiac function, reduced fibrosis and infarct size and increased angiogenesis (Kuhn et al. 2007).

Matrix proteins, matrix receptors or modulators of matrix deposition offer molecular targets that could be genetically engineered or otherwise modulated prior to stem cell infusion or injection. Up-regulation could be achieved directly via introduction of encoding cDNA or indirectly via inducers, e.g. periostin.

Improved cellular survival in the inflammatory myocardial environment after MI poses a challenge because typically 90% of all implanted and/or recruited cells die within a week (Laflamme and Murry 2005). Several principal strategies have been envisioned to improve stem cell intervention results and prognosis in MI, refractory angina pectoris or chronic heart failure. Because different stem cells tested, e.g. MSC, cardiac stem cell, endothelial progenitor cell, embryonic stem cells, multipotent adult stem cells, skeletal myoblasts or even hematopoietic stem cell, differ even the molecular details of these potential therapeutic strategies vary (Fig. 2.3; Penn and Mangi 2008). Cell surface receptors form potential targets for genetic engineering prior to stem cell delivery to target the cells correctly and effectively. Transmigration/extravasation of circulating stem cells could improve by up-regulation of receptors for stem cell homing factors (CXCR4/receptor for stromal-cell derived factor-1). Transient integrin expression (e.g. β_1 integrins) in delivered cells could improve stem cell migration and engraftment, whereas appropriate long-term integrin over-expression could alter stem cell differentiation towards the cardiac phenotype (Wu et al. 2006). Endothelial cell nitric oxide synthetase (eNOS)-mediated up-regulation of MMP-9 is estradiol dependent, suggesting a potential explanation for the better prognosis of MI in women (Iwakura et al. 2006). Because persistent ischemia leads to death of the transplanted cells, improved angiogenesis via angiogenic factors (VEGF, FGF-2, angiopoietins, TGF-β etc.) could enhance stem cell survival. Stem cell proliferation could be directly or indirectly be improved

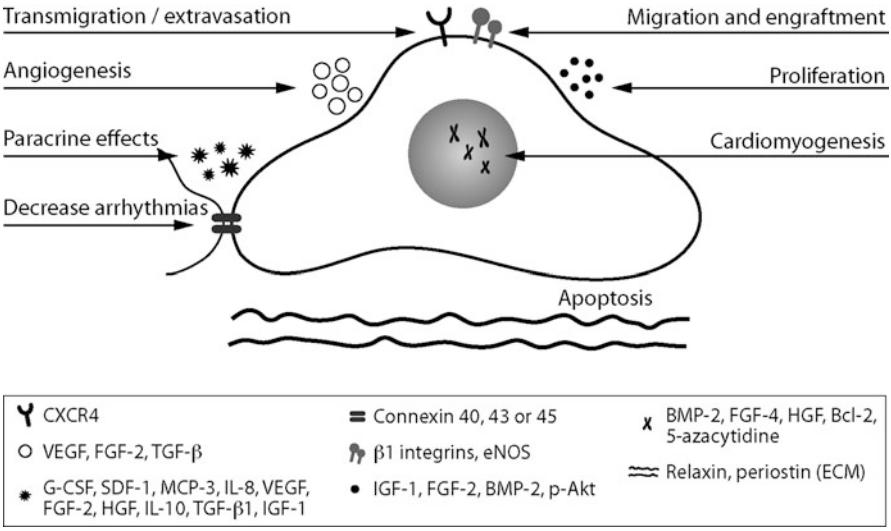


Fig. 2.3 Options to improve the beneficiail effects of mesenchymal stem cell therapies in heart disease include modulation of *1* Transmigration/extravasation, *2* migration and engraftment, *3* angiogenesis, *4* proliferation, *5* apoptosis, *6* stem cell produced paracrine factors (e.g. to help recruit endogenous resident stem cells), *7* cardiomyogenesis and *8* tendency to arrythmias (Modified from Penn and Mangi 2008)

via growth and trophic factors (FGF-2, IGF-1, BMP-2 and VEGF) or signal transduces (constitutively active serine/threonine protein kinase P-Akt, which mediates growth factor-associated cell survival signals). To improve ECM-cell survival signals and to prevent anoikis, the composition of the ECM could be modulated as to its tenascin-C (to be decreased), relaxin (to be increased) and periostin (to be increased) content (see above), but stem cells could also be embedded and added in epicardial collagen or laminin-1/Matrigel patches (Menasche 2007; Laflamme 2007) or the stem cell could be subjected anti-apoptotic gene transduction (of e.g. B-cell lymphoma 2=Bcl-2). Paracrine factors produced by stem cells can exert important effects, e.g. granulocyte-colony stimulating factor (G-CSF), stromal cell-derived factor 1 (SDF-1), monocyte chemoattractant protein-3 (MCP-3) and IL-8 are chemotactic and guide migration, VEGF, FGF-2 and HGF stimulate angiogenesis, FGF-2 stimulates proliferation, HGF reduces apoptosis, IL-10, TGF-β_1 and HGF exert anti-inflammatory effects and HGF and IGF-1 activate neighbouring resident stem cells. Further, MSCs decrease production of collagen type III, collagen type I and TIMP-1 (Crisostomo et al. 2008). Cardiomyogenic stimuli, like 5-azacytidine, BMP-2, FGF-4, HGF and transduction with Bcl-2 have tentatively been shown to induce differentiation of MSCs to cardiomyocytes, albeit this may only affect a small proportion of cells (Nesselmann et al. 2008). Finally, heart arrhythmias correlate with connexin protein expression (Mills et al. 2007). MSCs, which express connexins 40, 43 and 45 components of the gap junctions, have a significantly

decreased arrhythmogenic potential, whereas intramyocardially injected skeletal myoblasts with a high arrhythmogenic potential do not express any connexin proteins *in vivo*. Transplantation of myoblasts engineered to express connexin 43 decreased significantly ventricular tachycardias compared to transplantation with control skeletal myoblasts (Roell et al. 2007).

2.10.4 Heart and Valve Tissue Engineering

All four cardiac valves, aortic, mitral, pulmonary and tricuspidal, are attached to a fibrous supporting skeleton of tendon like tissue via a flexible hinge region composed of loose connective tissue intermingled with some muscle tissue. They have a similar layered and strong architectural structure. Heart valves are covered by endocardium composed of valvular endothelial cells (VEC), which are in contact with blood and which form a thin haemocompatible surface. The connective tissue matrix of the heart valve consists of three layers, which contain valvular interstitial cells (VIC) embedded in it. On the outflow surface is a dense and strong collagen-rich layer, which provides structural strength and stiffness. Collagen fibres withstand high tensile forces when taut, but buckling occurs upon compression. In the central core of the valve is the middle layer composed of GAG-rich, loose and spongy connective tissue absorbing shear and compression forces during the heart cycles. On the inflow surface is a layer rich in elastin, which extends in diastole and recoils in systole. In the aortic and pulmonary valves these three layers are called *fibrosa*, *spongiosa* and *ventricularis*, respectively. Heart valves are mostly avascular and have relatively poor repair ability although they adapt to their functional requirements as is for example seen in so called Ross operation, when the autologous pulmonary valve is transplanted to replace the aortic valve subjected to high flow and pressure circumstances (and a homograft is used to replace the pulmonary valve).

Healthy heart valves maintain unidirectional blood flow and act as backflow valves via an extraordinarily dynamic functional structure with sufficient strength and durability to withstand acute stresses and chronic fatigue-induced changes. The diastolic pattern of collagen alignment in the plane of the valve tissue is virtually complete already early after valve closure because diastolic collagen realignment occurs when the back pressure increases from 0 to 4 mgHg during the onset of cardiac diastole. Collagen crimp decreases rapidly as pressure is applied and is nearly completely (90%) lost at a transvalvular back pressure of 20 mgHg and only little further rearrangement occurs upon further increase of the pressure (Sacks and Yoganathan 2007).

Most VICs in the healthy valve are quiescent fibroblast-like cells, but they are highly plastic as shown by transition to their activation and matrix remodelling state in response to mechanical loading, injury or disease. Valvuloplasty or replacement of damaged cardiac valves by bioprosthesis or mechanical prostheses enhances quality of life and is often life saving. Due to the risk of thromboembolic complications

mediated via external coagulation cascade and in part integrin-mediated platelet aggregation and associated with abiotic mechanical prosthetic valves, long-term anticoagulation is used in spite of inherent risks for hemorrhagic complications. Therefore, valvuloplasties or bioprostheses, produced for example from glutaraldehyde-fixed porcine atrio-ventricular valves or bovine pericardium, are used even in half or more of valve replacements. Tissue degeneration leads gradually in the course of time to a failure of the bioprosthesis. Cumulative damage and dystrophic cuspal mineralization, initiated already by the devitalized VICs in the installed bioprosthesis, are the major causal mechanisms. Due to chemical fixation with glutaraldehyde the VICs and fibroblasts in the bioprosthesis die. Lack of remodelling capacity leads to irreversible and cumulative valve damage and impaired valve survival.

Attempts have been done to produce tissue-engineered heart valves by culturing autologous cells derived from vascular wall or bone marrow on biodegradable synthetic valve-like polymer scaffolds. An alternative tissue-engineering strategy, called guided tissue regeneration (Mendelson and Schoen 2006; Brody and Pandit 2007) uses an implanted scaffold composed of natural biomaterial or de-cellularized valve designed to attract circulating endothelial and other precursor cells and to provide a suitable environment for their adherence, growth, and differentiation. Attempts to produce engineered heart tissue have been done by mixing cardiac myocytes from neonatal Fischer 344 rats with liquid type I collagen (component of the interstitial stroma), Matrigel (basement membrane-like component), and serum (to enable the normal protein adsorption) containing culture medium (Zimmermann et al. 2002).

2.11 Tumour Extracellular Matrix

Cancer cells need to deal with extracellular matrix because they need space at the cost of the host tissue and without respect to normal tissue barriers pass them. At the same time, to be able to spread locally and to be able to form metastases after extravasation from blood or lymphatic vessels they need to be able to use host tissues as a platform for their integrin and non-integrin receptor-mediated or ameboid motility. They do not only use their own proteolytic machineries but are able to trick the host cells to pave them the way through the interstitial tissues, basement membranes and cellular endothelial or lymphatic endothelial barriers. Generalized MMP inhibitors have been a failure and the modern view of the proteinases regards them as often spatially strictly regulated signalling scissors, with important regulatory effects based on modulation of chemokines, cytokines, growth factors and their receptors.

In many cancers of epithelial origin epithelial mesenchymal transformation (EMT) is a central event. This term refers to dedifferentiation and detachment of the epithelial cells from the cell collective so that the detached cells become able to invade their immediate pericellular matrix, intravasate into the vascular or lymphatic

circulation, only to later extravasate again at secondary metastatic tumour sites to establish a new tumour. This involves primarily cadherin switch from the epithelial E-cadherin to the mesenchymal (and neuronal) N-cadherin, but also integrin-mediated cell-ECM events play a role. Engagement of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin receptors with collagen, typical for many connective tissue-embedded mesenchymal cells, down-regulates E-cadherin (Koenig et al. 2006). If at the same time discoidin domain receptor-1 for collagen is engaged, N-cadherin is simultaneously up-regulated (Shintani et al. 2008). This cadherin switch and epithelial-mesenchymal transformation can via E-cadherin repressor Snail1 increase expression of $\alpha_v\beta_3$ integrin, which we already know from above for its presence in activated endothelial cells and for its engagement in angiogenesis. This integrin is able to bind and focalize MMP-2 to the advancing edge of the cancer cells (Yilmaz and Christofori 2009), where also MT1-MMP is located. MT1-MMP can further focalize MMP-2 by using TIMP-2 as a bridge in ternary cell membrane-associated complexes. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_8$ bind pre-TGF-β_1 (LAP-TGF-β_1) released from degrading matrix depots, which TGF-β_1 upon local activation further contributes to cancer progression.

Both integrins, growth factor receptors and their ligands and membrane- or integrin-bound MMPs, which tend to act together, can be rapidly regulated via clathrin-mediated endocytosis (CME), raft/caveolar endocytosis (RCE) and macropinocytosis, which can rapidly transfer such factors to strategic locations or remove them to lysosomal degradation or recycle them back to cell membrane to regulate anchorage dependent growth, growth factor sensitivity and effects, and invasion into matrix (Ramsay et al. 2007). The balance between different endocytosis pathways is delicate: engagement of the CME can promote the above mentioned TGF-β_1 signalling, whereas endocytosis via RCE guides the same receptor-ligand complex towards lysosomal degradation (Di Guglielmo et al. 2003).

One key concept in modern cancer biology is the concept of cancer stem cells. Cancers have been treated and prognoses assessed based on the TNM classification, where the size of the tumour (T), growth in the regional lymph nodes (N) and tumour metastases (M) play an important role. In some forms of cancer it has been noticed that most of the tumour cells actually represent relatively well differentiated tumour cells, which to a large extent have lost their "malignant stemness": these tumour daughter cells can not easily form metastasis and can not be used to transfer cancer to cancer free animals. Among this multitude of cells reside a few cancer stem or parent cells, which maintain the tumour and send metastasis as described above. Because the immediate treatment responses and prognosis have been evaluated in terms of the size of the tumour and its regress upon treatment, drugs with a cytotoxic effect on the relatively "benign" and almost terminally differentiated cancer have been positively selected even though they would have little effect on the cancer stem cells *per se*. This can form a serious bias for the drug screening and selection process between various singular or combination treatments. Drugs with a predominant effect on the relatively benign tumour cells lead to a promising initial response but recurrence occurs upon longer follow up because the cancer stem cells soon produce more cancer cells, increase the tumour size by rapid division and aggressively send metastasis. In contrast, attention should be paid to the control of the cancer

stem cells: the initial response would be very modest, but gradually when the more differentiated cancer cells die of apoptosis or ischemic necrosis, the cancer would regress because the tumour can not now be maintained by asymmetric divisions of the cancer stem cells.

Degradation of ECM is normally tightly controlled because insufficient degradation would prevent normal cell migration and tissue remodelling, whereas excessive degradation would result in loss of attachment and anoikis and pathological destruction of connective tissue. MMPs are usually produced, stored, secreted, focalized (compartmentalized) and finally secreted as latent pro-enzymes, the activation of which is tightly regulated. Pro-MMP activation often involves plasminogen-plasmin conversion, which is regulated by tissue (tPA) and urokinase (uPA) type plasminogen activators as well as plasminogen activator inhibitors PAI-1 and -2 (Massova et al. 1998) in a number of pathological processes. MMPs can be activated via other MMPs, e.g. MMP-3 activates pro-MMP-1. Active site zinc can be released via oxygen radicals and some MMPs are activated by furins prior to secretion. Finally, the activity of MMPs is regulated by the availability of and avidity for different substrates and inhibitors and the stability of the enzyme.

To be able to move around, cells focus integrin receptors and MMPs at the leading edge of the cell in broad and flat actin-organized lamellipodia, which can send long, stiff and rod-like actin bundle-rich sensory filopodia as sensing cellular organs ahead of the main front. Much of currently available information on the motility of the cells has been obtained from 2D cell cultures, but *in vivo* cells usually reside in a 3D surrounding and produce instead podosomes or, in case of cancer cells, somewhat homologous invadopodia. These cellular extensions combine integrin- and non-integrin mediated adhesion to the surrounding matrix and highly organized proteolytic machinery with an actin-rich cytoskeletal core as the driving dynamic force. Invadopodia-associated integrin receptors $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_V\beta_3$ are suitable for attachment to laminin, fibronectin and vitronectin containing provisional matrix, focalization of the proteolytic, matrix degrading enzymes and signal transduction promoting the formation of invadopodia.

A key step in the regulation of MMPs is the conversion of the zymogen into the active proteinase. Because MMPs have the ability to act on extracellular matrix proteins, MMPs have been often implicated in tumour progression and metastasis as substances able to break down tissue barriers that otherwise restrain invasion (Coussens et al. 2002; Egeblad and Werb 2002). In cancer tissue several cell types, namely resident, inflammatory and tumour cells, express and regulate several different MMPs, which can either promote or restrain tissue destruction (Coussens et al. 2002; Egeblad and Werb 2002; Parks et al. 2004). Consequently, many pharmaceutical companies developed programs to target MMPs in cancer. Several drugs, designed to directly block the catalytic activity of MMPs, were tested even in phase III clinical trials, but none was effective (Coussens et al. 2002). The key shortcoming of the MMP inhibitor trials seems to be that they target the catalytic mechanism and lack MMP specificity (Coussens et al. 2002; Parks et al. 2004). They block the activity of all now known 24 MMPs (Table 2.6 “Matrix metalloproteinases”), but also the activity of the related ADAMs (a disintegrin and a

metalloproteinase) and ADAMTSs (a disintegrin and a metalloproteinase with a thrombospondin domain) as well as that of many other metalloenzymes. This is not an intelligent strategy because it is likely that the cells use several distinct mechanisms to balance and compartmentalize their metalloenzymes for remodelling, directed movement, pathological tissue destruction, including misguided pavement for invasion and metastasis for cancer cells. Further, MMPs play important roles as signalling scissors, anti-inflammatory and tissue protective roles because they can solubilise cell surface-bound cytokines, receptors, proteinases etc. More insight in the proMMPs activation and focalizaton is needed for selected blocking of the deleterious MMP-mediated processes at the same time when normal remodelling is retained.

2.12 Conclusion

It is concluded that ECM, although produced and degraded by cells, provides an important reference frame for cellular function as to structure and function. In particular, "immaterial" stimuli in form of biomechanical influences are mediated to cells via their matrix. Further, ECM it not a passive partner in cellular life. Part of its importance lies in the fact that the cells that produced it can die but the matrix script still stays after their apoptotic or necrotic death and disappearance from the scene. This is of particular importance in small and continuous scale in tissue remodelling and correction of the script and more drastically in tissue regeneration and particularly in repair when probably totally new textural information is produced: the progenitor and stem cells in particular can find important information from their predecessors and earlier cellular inhabitants of the matrix niche, from this rich source of information and growth factors so that the structures can be born and reborn over and over again, often in different and dynamic production-degradation phases in a process with a certain predetermined direction.

Acknowledgements This work was supported EU Matera grant "Bioactive Nanocomposite Constructs for Regeneration of Articular Cartilage", European Science Foundation "Regenerative Medicine" RNP, Danish Council for Strategic Research, Finska Läkaresällskapet, Orion Foundation and EU COST 533 Biotribology Action.

References

Ågren MS, Werthén M (2007) The extracellular matrix in wound healing: a closer look at therapeutics for chronic wounds. Int J Low Extrem Wounds 6:82–97

Ågren MS, Eaglstein WH, Ferguson MW, Harding KG, Moore K, Saarialho-Kere UK, Schultz GS (2000) Causes and effects of the chronic inflammation in venous leg ulcers. Acta Derm Venereol Suppl 210:3–17

Alford JW, Cole BJ (2005) Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. Am J Sports Med 33:295–306

Allemann F, Mizuno S, Eid K, Yates KE, Zaleske D, Glowacki J (2001) Effects of hyaluronan on engineered articular cartilage extracellular matrix gene expression in 3-dimensional collagen scaffolds. J Biomed Mater Res 55:13–19

Alsalameh S, Amin R, Gemba T, Lotz M (2004) Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. Arthritis Rheum 50:1522–1532

Amalinei C, Caruntu I-D, Balan RA (2007) Biology of metalloproteinases. Rom J Morphol Embryol 48:323–334

Angata T, Brinkman-van der Linden ECM (2002) I-type lectins. Biochim Biophys Acta 1572:294–316

Arikawa T, Matsukawa A, Watanabe K, Sakata KM, Seki M, Nagayama M, Takeshita K, Ito K, Niki T, Oomizu S, Shinonaga R, Saita N, Hirashima M (2009) Galectin-9 accelerates transforming growth factor beta3-induced differentiation of human mesenchymal stem cells to chondrocytes. Bone 44:849–857

Askari A, Brennan ML, Zhou X, Drinko J, Morehead A, Thomas JT, Topol EJ, Hazen SL, Penn MS (2003) Myeloperoxidase and plasminogen activator inhibitor-1 play a central role in ventricular remodeling after myocardial infarction. J Exp Med 197:615–624

Aszódi A, Legate KR, Nakchbandi I, Fässler R (2006) What mouse mutants teach us about extracellular matrix function. Annu Rev Cell Dev Biol 22:591–621

Aumailley M, Bruckner-Tuderman L, Carter WG et al (2005) A simplified laminin nomenclature. Mini review. Matrix Biol 24:326–332

Badylak SF (2002) The extracellular matrix as a scaffold for tissue reconstruction. Semin Cell Dev Biol 13:377–383

Bani D, Nistri S, Formigli L, Meacci E, Francini F, Zecchi-Orlandini S (2009) Prominent role of relaxin in improving postinfarction heart remodeling. Clues from in vivo and in vitro studies with genetically engineered relaxin-producing myoblasts. Ann N Y Acad Sci 1160:269–277

Battiwalla M, Hematti P (2009) Mesenchymal stem cells in hematopoietic stem cell transplantation. Cytotherapy 11:503–515

Beauvais DM, Ell BJ, McWhorter AR, Rapraeger AC (2009) Syndecan-1 regulates $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin activation during angiogenesis and is blocked by synstatin, a novel peptide inhibitor. J Exp Med 206:691–705

Bernfield M, Kokenyesi R, Kato M, Hinkes MT, Spring J, Gallo RL, Lose EJ (1992) Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. Ann Rev Biol 8:365–393

Borza DB, Bondar O, Ninomiya Y, Sado Y, Naito I, Todd P, Hudson BG (2001) The NC1 domain of collagen IV encodes a novel network composed of the alpha 1, alpha 2, alpha 5, and alpha 6 chains in smooth muscle basement membranes. J Biol Chem 276:28532–28540

Boutaud A, Borza DB, Bondar O, Gunwar S, Netzer KO, Singh N, Ninomiya Y, Sado Y, Noelken ME, Hudson BG (2000) Type IV collagen of the glomerular basement membrane. Evidence that the chain specificity of network assembly is encoded by the noncollagenous NC1 domains. J Biol Chem 275:30716–30724

Brody S, Pandit A (2007) Approaches to heart valve tissue engineering scaffold design. J Biomed Mater Res Part B Appl Biomater 83:16–43

Brooks PC, Clark RAF, Cheresh DA (1994) Requirement of vascular integrin αVβ3 for angiogenesis. Science 264:569–571

Bullard KM, Longaker MT, Lorenz HP (2003) Fetal wound healing: current biology. World J Surg 27:54–61

Canty EG, Kadler KE (2005) Procollagen trafficking, processing and fibrillogenesis. J Cell Sci 118:1341–1353

Capito RM, Spector M (2003) Scaffold-based articular cartilage repair. IEEE Eng Med Biol Mag Q Mag Eng Med Biol Soc 22:42–50

Clark RAF (1995) Wound repair: overview and general considerations. In: The molecular and cellular biology of wound repair, 2nd edn. Plenum Press, New York, pp 513–560

Cosgrove D, Meehan DT, Grunkemeyer JA, Kornak JM, Sayers R, Hunter WJ, Samuelson GC (1996) Collagen COL4A3 knockout: a mouse model for autosomal Alport syndrome. Genes Dev 10:2981–2992

Coussens LM, Fingleton B, Matrisian LM (2002) Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science 295:2387–2392
Creemers EE, Cleutjens JP, Smits JF, Daemen MJ (2001) Matrix metalloproteinase inhibition after myocardial infarction: a new approach to prevent heart failure? Circ Res 89:201–210
Crisostomo PR, Markel TA, Wang Y, Meldrum DR (2008) Surgically relevant aspects of stem cell paracrine effects. Surgery 143:577–581
Cui F-Z, Li Y, Ge J (2007) Self-assembly of mineralized collagen composites. Mater Sci Eng R 57:1–27
Davis GE, Senger DR (2005) Endothelial extracellular matrix. Biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ Res 97:1093–1107
Deten A, Holzl A, Leicht M, Barth W, Zimmer HG (2001) Changes in extracellular matrix and in transforming growth factor beta isoforms after coronary artery ligation in rats. J Mol Cell Cardiol 33:1191–1207
Deten A, Volz HC, Briest W, Zimmer HG (2002) Cardiac cytokine expression is upregulated in the acute phase after myocardial infarction. Experimental studies in rats. Cardiovasc Res 55:329–340
Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL (2003) Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. Nat Cell Biol 5:410–421
Docheva D, Popov C, Mutschler W, Schieker M (2007) Human mesenchymal stem cells in contact with their environment: surface characteristics and the integrin system. J Cell Mol Med 11:21–38
Doe CQ, Bowerman B (2001) Asymmetric cell division: fly neuroblast meets worm zygote. Curr Opin Cell Biol 13:68–75
East L, Isacke CM (2002) The mannose receptor family. Biochim Biophys Acta 1572:364–386
Eckes B, Kessler D, Aumailley M, Krieg T (1999) Interactions of fibroblasts with the extracellular matrix: implications for the understanding of fibrosis. Springer Semin Immunopathol 21:415–429
Egeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2:161–174
Egles C, Claudepierre T, Manglapus MK, Champliaud MF, Brunken WJ, Hunter DD (2007) Laminins containing the beta2 chain modulate the precise organization of CNS synapses. Mol Cell Neurosci 34:388–398
Ekblom P, Lonai P, Talts JF (2003) Expression and biological role of laminin-1. Matrix Biol J Int Soc Matrix Biol 22:35–47
Eklund L, Piuhola J, Komulainen J, Sormunen R, Ongvarrasopone C, Fässler R, Muona A, Ilves M, Ruskoaho H, Takala TE, Pihlajaniemi T (2001) Lack of type XV collagen causes a skeletal myopathy and cardiovascular defects in mice. Proc Natl Acad Sci U S A 98:1194–1199
Ellis SJ, Tanantzapf G (2010) Integrin-mediated adhesion and stem-cell-niche interactions. Cell Tissue Res 339:121–130
Engler AJ, Sen S, Sweeney HL, Discher DE (2006) Matrix elasticity directs stem cell lineage specification. Cell 126:677–689
Fanjul-Fernández M, Folgueras AR, Cabrera S, López-Otin C (2010) Matrix metalloproteinases: evolution, gene regulation and functional analysis in mouse models. Biochim Biophys Acta 1803:3–19
Fawcett JW, Asher RA (1999) The glial scar and central nervous system repair. Brain Res Bull 49:377–391
Fortier LA, Mohammed HO, Lust G, Nixon AJ (2002) Insulin-like growth factor-I enhances cell-based repair of articular cartilage. J Bone Jt Surg Br Vol 84-B:276–288
Fox JW, Mayer U, Nischt R, Aumailley M, Reinhardt D, Wiedemann H, Mann K, Timpl R, Krieg T, Engel J, Chu M-L (1991) Recombinant nidogen consists of three globular domains and mediates binding of laminin to collagen type IV. EMBO J 10:3137–3146
Fox JM, Ghamberlain G, Ashton BA, Middleton J (2007) Recent advances into the understanding of mesenchymal stem cell trafficking. Br J Haematol 137:491–502
Fukai N, Eklund L, Marneros AG, Oh SP, Keene DR, Tamarkin L, Niemelä M, Ilves M, Li E, Pihlajaniemi T, Olsen BR (2002) Lack of collagen XVIII/endostatin results in eye abnormalities. EMBO J 21:1535–1544

Getgood A, Brooks R, Fortier L, Rushton N (2009) Articular cartilage tissue engineering: today's research, tomorrow's practice? J Bone Jt Surg Br Vol 91:565–576

Glowacki J, Mizuno S (2007) Collagen scaffolds for tissue engineering. Biopolymers 89:338–344

Gohring W, Sasaki T, Heldin CH, Timpl R (1998) Mapping of the binding of platelet-derived growth factor to distinct domains of the basement membrane proteins BM-40 and perlecan and distinction from the BM-40 collagen-binding epitope. Eur J Biochem 255:60–66

Gray H, Bannister LH, Berry MM, Williams PL (1995) Gray's anatomy: the anatomical basis of medicine and surgery. Churchill Livingstone, Edinburgh

Gronthos S, Simmons PJ, Graves SE, Robey PG (2001) Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. Bone 28:174–181

Guo T, Zhao J, Chang J, Ding Z, Hong H, Chen J, Zhang J (2006) Porous chitosan-gelatin scaffold containing plasmid DNA encoding transforming growth factor-beta1 for chondrocytes proliferation. Biomaterials 27:1095–1103

Hayes AJ, Tudor D, Nowell MA, Caterson B, Hughes CE (2008) Chondroitin sulphate sulfation motifs as putative biomarkers for isolation of articular cartilage progenitor cells. J Histochem Cytochem 56:125–138

Heino J (2007) The collagen family members as cell adhesion proteins. BioEssays News Rev Mol Cell Dev Biol 29:1001–1010

Heino J, Käpylä J (2009) Cellular receptors of extracellular matrix molecules. Curr Pharm Des 15:1309–1317

Heinola T, Kouri V-P, Clarijs P, Ciferska H, Sukura A, Salo J, Konttinen YT (2010) High mobility group box-1 (HMGB-1) in osteoarthritic cartilage. Clin Exp Rheumatol 28:511–518

Herrick SE, Sloan P, McGurk M, Freak L, McCollum CN, Ferguson MW (1992) Sequential changes in histologic pattern and extracellular matrix deposition during the healing or chronic venous ulcers. Am J Pathol 141:1085–1095

Herrick SE, Ireland GW, Simon D, McCollum CN, Ferguson MW (1996) Venous ulcer fibroblasts compared with normal fibroblasts show differences in collagen but not fibronectin production under both normal and hypoxic conditions. J Investig Dermatol 106:187–193

Hodde JP, Johnson CE (2007) Extracellular matrix as a strategy for treating chronic wounds. Am J Clin Dermatol 8:61–66

Hoemann CD, Sun J, McKee MD, Chevrier A, Rossomacha E, Rivard GE, Hurtig M, Buschmann MD (2007) Chitosan-glycerol phosphate/blood implants elicit hyaline cartilage repair integrated with porous subchondral bone in microdrilled rabbit defects. Osteoarthr Cartil 15:78–89

Hudson BG (2004) The molecular basis of Goodpasture and Alport syndromes: beacons for the discovery of the collagen IV family. J Am Soc Nephrol 15:2514–2527

Hudson BG, Tryggvason K, Sundaramoorthy M, Neilson EG (2003) Alport's syndrome, Goodpasture's syndrome, and type IV collagen. N Engl J Med 348:2543–2556

Hutmacher DW (2000) Scaffolds in tissue engineering bone and cartilage. Biomaterials 21:2529–2543

Hutmacher DW, Kirsch A, Ackermann KL, Huerzeler MB (1998) Matrix and carrier materials for bone growth factors – state of the art and future perspectives. In: Stark GB, Horch R, Tancos E (eds) Biological matrices and tissue reconstruction. Springer, Heidelberg, pp 197–206

Hwang MW, Matsumori A, Furukawa Y, Ono K, Okada M, Iwasaki A, Hara M, Miyamoto T, Touma M, Sasayama S (2001) Neutralization of interleukin-1beta in the acute phase of myocardial infarction promotes the progression of left ventricular remodeling. J Am Coll Cardiol 38:1546–1553

Imanaka-Yoshida K, Hiroe M, Nishikawa T, Ishiyama S, Shimojo T, Ohta Y, Sakakura T, Yoshida T (2001) Tenascin-C modulates adhesion of cardiomyocytes to extracellular matrix during tissue remodeling after myocardial infarction. Lab Investig J Tech Methods Pathol 81:1015–1024

Ip JE, Wu Y, Huang J, Zhang L, Pratt RE, Dzau VJ (2007) Mesenchymal stem cells use integrin beta1 not CXC chemokine receptor 4 for myocardial migration and engraftment. Mol Biol Cell 18:2873–2882

Irwin CR, Myrillas T, Smyth M, Doogan J, Rice C, Schor SL (1998) Regulation of fibroblast-induced collagen gel contraction by interleukin-1b. J Oral Pathol Med 27:255–259

Iwakura A, Shastry S, Luedemann C, Hamada H, Kawamoto A, Kishore R, Zhu Y, Qin G, Silver M, Thorne T, Eaton L, Masuda H, Asahara T, Losordo DW (2006) Estradiol enhances recovery after myocardial infarction by augmenting incorporation of bone marrow-derived endothelial progenitor cells into sites of ischemia-induced neovascularization via endothelial nitric oxide synthase-mediated activation of matrix metalloproteinase-9. Circulation 113:1605–1614

Jäälinoja J (2007) The structure and function of normal and mutated collagen IX. Thesis work. Oulu University Press, Oulu

Jones JC, Dehart GW, Gonzales M, Goldfinger LE (2000) Laminins: an overview. Microsc Res Tech 51:211–213

Juncosa-Melvin N, Shearn JT, Boivin GP, Gooch C, Galloway MT, West JR, Nirmalanandhan VS, Bradica G, Butler DL (2006) Effects of mechanical stimulation on the biomechanics and histology of stem cell-collagen sponge constructs for rabbit patellar tendon repair. Tissue Eng 12:2291–2300

Kalluri R (2003) Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer 3:422–433

Karadag A, Fisher LW (2006) Bone sialoprotein enhances migration of bone marrow stromal cells through matrices by bridging MMP-2 to alpha(v)beta3-integrin. J Bone Miner Res 21:1627–1636

Kato Y, Nishikawa T, Kawakishi S (1995) Antioxidative activity of tetrahydrocurcuminoids. Photochem Photobiol 61:367–372

Katsuragi N, Morishita R, Nakamura N, Ochiai T, Taniyama Y, Hasegawa Y, Kawashima K, Kaneda Y, Ogihara T, Sugimura K (2004) Periostin as a novel factor responsible for ventricular dilation. Circulation 110:1806–1813

Keating A (2012) Mesenchymal stromal cells: new directions. Cell Stem Cell 10:706–716

Kefalides NA (1966) A collagen of unusual composition and a glycoprotein isolated from canine glomerular basement membrane. Biochem Biophys Res Commun 22:26–32

Khang D, Park GE, Webster TJ (2008) Enhanced chondrocyte densities on carbon nanotube composites: the combined role of nanosurface roughness and electrical stimulation. J Biomed Mater Res Part A 86-A:253–260

Khoshnoodi J, Pedchenko V, Hudson BG (2008) Mammalian collagen IV. Microsc Res Tech 71:357–370

Kikkawa Y, Moulson CL, Virtanen I, Miner JH (2002) Identification of the binding site for the Lutheran blood group glycoprotein on laminin alpha 5 through expression of chimeric laminin chains in vivo. J Biol Chem 277:44864–44869

Kleinman HK, Klebe RJ, Martin GR (1981) Role of collagenous matrices in the adhesion and growth of cells. J Cell Biol 88:473–485

Knudson W, Casey B, Nishida Y, Eger W, Kuettner KE, Knudson CB (2000) Hyaluronan oligosaccharides perturb cartilage matrix homeostasis and induce chondrocytic chondrolysis. Arthritis Rheum 43:1165–1174

Ko GJ, Kang YS, Han SY, Lee MH, Song HK, Han KH, Kim HK, Han JY, Cha DR (2008) Pioglitazone attenuates diabetic nephropathy through an anti-inflammatory mechanism in type 2 diabetic rats. Nephrol Dial Transplant 23:2750–2760

Koenig A, Mueller C, Hasel C, Adler G, Menke A (2006) Collagen type I induces disruption of E-cadherin-mediated cell-cell contacts and promotes proliferation of pancreatic carcinoma cells. Cancer Res 66(9):4662–4671

Kohfeldt E, Sasaki T, Gohring W, Timpl R (1998) Nidogen-2: a new basement membrane protein with diverse binding properties. J Mol Biol 282:99–109

Kuhn B, del Monte F, Hajjar RJ, Chang YS, Lebeche D, Arab S, Keating MT (2007) Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. Nat Med 13:962–969

Kuo CK, Tuan RS (2008) Mechanoactive tenogenic differentiation of human mesenchymal stem cells. Tissue Eng Part A 14:1615–1627

Kvist AP, Latvanlehto A, Sund M, Eklund L, Väisänen T, Hägg P, Sormunen R, Komulainen J, Fässler R, Pihlajaniemi T (2001) Lack of cytosolic and transmembrane domains of type XIII collagen results in progressive myopathy. Am J Pathol 159:1581–1592

Laflamme MA (2007) Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25:1015–1024
Laflamme MA, Murry CE (2005) Regenerating the heart. Nat Biotechnol 23:845–856
Laine M, Virtanen I, Salo T, Konttinen YT (2004) Segment-specific but pathologic laminin isoform profiles in human labial salivary glands of patients with Sjögren's syndrome. Arthritis Rheum 50:3968–3973
Laine M, Virtanen I, Porola P, Rotar Z, Rozman B, Poduval P, Konttinen YT (2008) Acinar epithelial cell laminin-receptors in labial salivary glands in Sjögren's syndrome. Clin Exp Rheumatol 26:807–813
Laurens N, Engelse MA, Jungerius C, Löwik CW, van Hinsbergh VWM, Koolwijk P (2009) Single and combined effects of avb3- and a5b1-integrins on capillary tube formation in a human fibrinous matrix. Angiogenesis 12:275–285
Leitinger B, Hohenester E (2007) Mammalian collagen receptors. Matrix Biol J Int Soc Matrix Biol 26:146–155
Li S, Liquari P, McKee KK, Harrison D, Patel R, Lee S, Yurchenco PD (2005a) Laminin-sulfatide binding initiates basement membrane assembly and enables receptor signaling in Schwann cells and fibroblasts. J Cell Biol 169:179–189
Li WJ, Tuli R, Huang X, Laquerriere P, Tuan RS (2005b) Multilineage differentiation of human mesenchymal stem cells in a three-dimensional nanofibrous scaffold. Biomaterials 26:5158–5166
Li G, Zhang XA, Wang H, Wang X, Meng CL, Chan CY, Yew DT, Tsang KS, Li K, Tsai SN, Ngai SM, Han ZC, Lin MC, He ML, Kung HF (2009) Comparative proteomic analysis of mesenchymal stem cells derived from human bone marrow, umbilical cord, and placenta: implication in the migration. Proteomics 9:20–30
Litvin J, Blagg A, Mu A, Matiwala S, Montgomery M, Berretta R, Houser S, Margulies K (2006) Periostin and periostin-like factor in the human heart: possible therapeutic targets. Cardiovas Pathol Off J Soc Cardiovasc Pathol 15:24–32
Lu L, Zhu X, Valenzuela RG, Currier BL, Yaszemski MJ (2001) Biodegradable polymer scaffolds for cartilage tissue engineering. Clin Orthop Relat Res 391(Suppl):S251–S270
Ma GF, Ali A, Verzijl N, Hanemaaijer R, TeKoppele J, Konttinen YT, Salo J (2006) Increased collagen degradation around loosened total hip replacement implants. Arthritis Rheum 54:2928–2933
Maeshima Y, Yerramalla UL, Dhanabal M, Holthaus KA, Barbashov S, Kharbanda S, Reimer C, Manfredi M, Dickerson WM, Kalluri R (2001) Extracellular matrix-derived peptide binds to alpha(v)beta(3) integrin and inhibits angiogenesis. J Biol Chem 276:31959–31968
Maeshima Y, Sudhakar A, Lively JC, Ueki K, Kharbanda S, Kahn CR, Sonenberg N, Hynes RO, Kalluri R (2002) Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. Science 295:140–143
Maitra N, Flink IL, Bahl JJ, Morkin E (2000) Expression of α and β integrins during terminal differentiation of cardiomyocytes. Cardiovasc Res 47:715–725
Martinez-Lemus LA, Wu X, Wilson E, Hill MA, Davis GE, Davis MJ, Meininger GA (2003) Integrins as unique receptors for vascular control. J Vasc Res 40:211–233
Martinez-Pomares L, Wienke D, Stillion R, McKenzie EJ, Arnold JN, Harris J, McGreal E, Sim RB, Isacke CM, Gordon S (2006) Carbohydrate-independent recognition of collagens by the macrophage mannose receptor. Eur J Immunol 36:1074–1082
Massova I, Kotra LP, Fridman R, Mobashery S (1998) Matrix metalloproteinases: Structure evolution and diversification. FASEB J Off Publ Fed Am Soc Exp Biol 12:1075–1095
McKee KK, Harrison D, Capizzi S, Yurchenco PD (2007) Role of laminin terminal globular domains in basement membrane assembly. J Biol Chem 282:21437–21447
Menasche P (2007) Skeletal myoblasts as a therapeutic agent. Prog Cardiovasc Dis 50:7–17
Menasche P (2009) Cell-based therapy for heart disease: a clinically oriented perspective. Mol Ther J Am Soc Gene Ther 17(5):758–766
Mendelson K, Schoen FJ (2006) Heart valve tissue engineering: concepts, approaches, progress, and challenges. Ann Biomed Eng 34:1799–1819

Menke NB, Ward KR, Witten TM, Bonchev DG, Diegelmann RF (2007) Impaired wound healing. Clin Dermatol 25:19–25

Messaritou G, East L, Roghi C, Isacke CM, Yarwood H (2009) Mebrane type-1 matrix metalloproteinase activity is regulated by the endocytic collagen receptor Endo180. J Cell Sci 122:4042–4048

Mias C, Lairez O, Trouche E, Roncalli J, Calise D, Seguelas MH, Ordener C, Piercecchi-Marti MD, Auge N, Salvayre AN, Bourin P, Parini A, Cussac D (2009) Mesenchymal stem cells promote matrix metalloproteinase secretion by cardiac fibroblasts and reduce cardiac ventricular fibrosis after myocardial infarction. Stem Cells 27:2734–2743

Mills WR, Mal N, Kiedrowski MJ, Unger R, Forudi F, Popovic ZB, Penn MS, Laurita KR (2007) Stem cell therapy enhances electrical viability in myocardial infarction. J Mol Cell Cardiol 42:314

Mizuno S, Glowacki J (1996) Three-dimensional composite of demineralized bone powder and collagen for in vitro analysis of chondroinduction of human dermal fibroblasts. Exp Cell Res 227:89–97

Morais Freitas V, Nogueira da Gama de Souza L, Cyreno Oliveira E, Furuse C, Cavalcanti de Araújo V, Gastaldoni Jaeger R (2007) Malignancy-related 67 kDa laminin receptor in adenoid cystic carcinoma. Effect on migration and beta-catenin expression. Oral Oncol 43:987–998

Murphy G, Nagase H (2008) Progress in matrix metalloproteinase research. Mol Asp Med 29:290–308

Myllyharju J, Kivirikko KI (2001) Collagens and collagen-related diseases. Ann Med 33:7–21

Naumann A, Dennis JE, Awadallah A, Carrino DA, Mansour JM, Kastenbauer E, Caplan AI (2002) Immunochemical and mechanical characterization of cartilage subtypes in rabbit. J Histochem Cytochem 50:1049–1058

Nesselmann C, Ma N, Bieback K, Wagner W, Ho A, Konttinen YT, Zhang H, Hinescu ME, Steinhoff G (2008) Mesenchymal stem cells and cardiac repair. J Cell Mol Med 12:1795–1810

Nichols JE, Cortiella J, Lee J, Niles JA, Cuddihy M, Wang S, Bielitzki J, Cantu A, Mlcak R, Valdivia E, Yancy R, McClure ML, Kotov NA (2009) In vitro analog of human bone marrow from 3D scaffolds with biomimetic inverted colloidal crystal geometry. Biomaterials 30:1071–1079

Nie D (2010) Cancer stem cell and niche. Front Biosci 2:184–193

Nisato RE, Tille JC, Jonczyk A, Goodman SL, Pepper MS (2003) Alphav beta 3 and alphav beta 5 integrin antagonists inhibit angiogenesis in vitro. Angiogenesis 6:105–119

Nishioka T, Suzuki M, Onishi K, Takakura N, Inada H, Yoshida T, Hiroe M, Imanaka-Yoshida K (2007) Eplerenone attenuates myocardial fibrosis in the angiotensin II-induced hypertensive mouse: involvement of tenascin-C induced by aldosterone-mediated inflammation. J Cardiovasc Pharmacol 49:261–268

O'Donoghue K, Fisk NM (2004) Fetal stem cells. Best Pract Res Clin Obstet Gynaecol 18:853–875

Ohga S, Shikata K, Yozai K, Okada S, Ogawa D, Usui H, Wada J, Shikata Y, Makino H (2007) Thiazolidinedione ameliorates renal injury in experimental diabetic rats through anti-inflammatory effects mediated by inhibition of NF-kappaB activation. Am J Physiol Ren Physiol 292:F1141–F1150

Park JB, Lakes RS (1992) Biomaterials: an introduction. Plenum, New York

Parks WC, Wilson CL, Lopez-Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 4:617–629

Patarroyo M, Tryggvason K, Virtanen I (2002) Laminins isoforms in tumor invasion, angiogenesis and metastasis. Semin Cancer Biol 12:197–207

Paweletz N, Knierim M (1989) Tumor related angiogenesis. Crit Rev Oncol/Hematol 9:197–242

Penn MS, Mangi AA (2008) Genetic enhancement of stem cell engraftment, survival, and efficacy. Circ Res 102(12):1471–1482

Perna AM, Masini E, Nistri S, Bani ST, Bigazzi M, Bani D (2005) Human recombinant relaxin reduces heart injury and improves ventricular performance in a swine model of acute myocardial infarction. Ann N Y Acad Sci 1041:431–433

Pommerenke H, Schreiber E, Dürr F, Nebe B, Möller W, Rychly J (1996) Stimulation of integrin receptors using a magnetic drag force device induces an intracellular free calcium response. Eur J Cell Biol 70:157–164

Poole CA (1992) Chondrons, the chondrocyte and its pericellular microenvironment. In: Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds) Articular cartilage and osteoarthritis. Raven, New York, pp 201–220

Rabinovich GA, Rubinstein N, Toscano MA (2002) Role of galectins in inflammatory and immunomodulatory processes. Biochim Biophys Acta 1572:274–284

Ramsay AG, Marshall JF, Hart IR (2007) Integrin trafficking and its role in cancer metastasis. Cancer Metastasis Rev 26:567–578

Reichenberger E, Baur S, Sukotjo C, Olsen BR, Karimbux NY, Nishimura I (2000) Collagen XII mutation disrupts matrix structure of periodontal ligament and skin. J Dent Res 79:1962–1968

Rho JY, Kuhn-Spearing L, Zioupos P (1998) Mechanical properties and the hierarchical structure of bone. Med Eng Phys 20:92–102

Ries C, Egea V, Karow M, Kolb H, Jochum M, Neth P (2007) MMP-2, T1-MMP, and TIMP-2 are essential for the invasive capacity of human esenchymal stem cells: Differential regulation by inflammatory cytokines. Blood 109:4055–4063

Risteli J, Bachinger HP, Engel J, Furthmayr H, Timpl R (1980) 7-S collagen: characterization of an unusual basement membrane structure. Eur J Biochem 108:239–250

Robles DT, Berg D (2007) Abnormal wound healing: keloids. Clin Dermatol 25:26–32

Roell W, Lewalter T, Sasse P, Tallini YN, Choi BR, Breitbach M, Doran R, Becher UM, Hwang SM, Bostani T, von Maltzahn J, Hofmann A, Reining S, Eiberger B, Gabris B, Pfeifer A, Welz A, Willecke K, Salama G, Schrickel JW, Kotlikoff MI, Fleischmann BK (2007) Engraftment of connexin 43-expressing cells prevents post-infarct arrhythmia. Nature 450:819–824

Rüster B, Göttig S, Ludwig RJ, Bistrian R, Müller S, Seifried E, Gille J, Henschler R (2006) Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. Blood 108:3938–3944

Sacks MS, Yoganathan AP (2007) Heart valve function: a biomechanical perspective. Philos Trans R Soc Lond Ser B Biol Sci 362:1369–1391

Sasaki K, Takagi M, Konttinen YT, Sasaki A, Tamaki Y, Ogino T, Santavirta S, Salo J (2007) Upregulation of matrix metalloproteinase (MMP)-1 and its activator MMP-3 of human osteoblast by uniaxial cyclic stimulation. J Biomed Mater Res Part B Appl Biomater 80:491–498

Sato A, Aonuma K, Imanaka-Yoshida K, Yoshida T, Isobe M, Kawase D, Kinoshita N, Yazaki Y, Hiroe M (2006) Serum tenascin-C might be a novel predictor of left ventricular remodeling and prognosis after acute myocardial infarction. J Am Coll Cardiol 47:2319–2325

Schultz GS, Ladwig G, Wysocki A (2005) Extracellular matrix: review of its roles in acute and chronic wounds. World Wide Wounds. www.worldwidewounds.com/2005/august/Schultz/Extrace-Matric-Acute-Chronic-Wounds.html

Sekiya I, Colter DC, Prockop DJ (2001) BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells. Biochem Biophys Res Commun 284:411–418

Shanbhag S, Woo Lee J, Kotov N (2005) Diffusion in three-dimensionally ordered scaffolds with inverted colloidal crystal geometry. Biomaterials 26:5581–5585

Shimaoka M, Takagi J, Springer TA (2002) Conformational regulation of integrin structure and function. Ann Rev Biophys Biomol Struct 31:485–516

Shimazaki M, Nakamura K, Kii I, Kashima T, Amizuka N, Li M, Saito M, Fukuda K, Nishiyama T, Kitajima S, Saga Y, Fukayama M, Sata M, Kudo A (2008) Periostin is essential for cardiac healing after acute myocardial infarction. J Exp Med 205:295–303

Shintani Y, Fukumoto Y, Chaika N, Svoboda R, Wheelock MJ, Johnson KR (2008) Collagen I-mediated upregulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor I. J Cell Biol 180:1277–1289

Silva R, D'Amico G, Hodivala-Dilke KM, Reynolds LE (2008) Integrins. The keys to unlocking angiogenesis. Arterioscler Thromb Vasc Biol 28:1703–1713

Solchaga LA, Goldberg VM, Caplan AI (2001) Cartilage regeneration using principles of tissue engineering. Clin Orthop Relat Res 391S:S161–S170

Steffensen B, Häkkinen L, Larjava H (2001) Proteolytic events of wound-healing – coordinated intractions among matrix metalloproteinases (MMPs), integrins, and extracellular matrix molecules. Crit Rev Oral Biol Med 12:373–398

Steingen C, Brenig F, Baumgartner L, Schmidt J, Schmidt A, Bloch W (2008) Characterization of key mechanisms in transmigration and invasion of mesenchymal stem cells. J Mol Cell Cardiol 44:1072–1084

Steinmeyer J, Konttinen YT (2006) Oral treatment options for degenerative joint disease – presence and future. Adv Drug Deliv Rev 58:168–211

Sun M, Opavsky A, Stewart DJ, Rabinovtich M, Dawood F, Wen WH, Liu PP (2003) Temporal response and localization of integrins beta-1 and beta-2 in the heart following myocardial infarction: regulation by cytokines. Circulation 107:1046–1052

Sund M, Ylönen R, Tuomisto A, Sormunen R, Tahkola J, Kvist AP, Kontusaari S, Autio-Harmainen H, Pihlajaniemi T (2001) Abnormal adherence junctions in the heart and reduced angiogenesis in transgenic mice overexpressing mutant type XIII collagen. EMBO J 20:5153–5164

Sunk IG, Bobacz K, Hofstaetter JG, Amoyo L, Soleiman A, Smolen J, Xu L, Li Y (2007) Increased expression of discoidin domain receptor 2 is linked to the degree of cartilage damage in human knee joints: a potential role in osteoarthritis pathogenesis. Arthritis Rheum 56:3685–3692

Takagi M (1996) Neutral proteinases and their inhibitors in the loosening of total hip prosthesis. Acta Orthop Scand 67(Suppl 271):1–29

Tamaoki M, Imanaka-Yoshida K, Yokoyama K, Nishioka T, Inada H, Hiroe M, Sakakura T, Yoshida T (2005) Tenascin-C regulates recruitment of myofibroblasts during tissue repair after myocardial injury. Am J Pathol 167:71–80

Taylor D (2007) Fracture and repair of bone: a multiscale problem. J Mater Sci 42:8911–8918

Tzu J, Marinkovich MP (2008) Bridging structure with function: structural, regulatory, and developmental role of laminins. Int J Biochem Cell Biol 40:199–214

Uygun BE, Stojsih SE, Matthew HW (2009) Effects of immobilized glycosaminoglycans on the proliferation and differentiation of mesenchymal stem cells. Tissue Eng Part A 15:3499–3512

van der Kraan PM, Buma P, van Kuppevelt T, van den Berg WB (2002) Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. Osteoarthr Cartil 10:631–637

van Osch GJ, Brittberg M, Dennis JE, Bastiaansen-Jenniskens YM, Erben RG, Konttinen YT, Luyten FP (2009) Cartilage repair: past and future – lessons for regenerative medicine. J Cell Mol Med 13:792–810

Veilleux N, Spector M (2005) Effects of FGF-2 and IGF-1 on adult canine articular chondrocytes in type II collagen-glycosaminoglycan scaffolds in vitro. Osteoarthr Cartil 13:278–286

Veit G, Kobbe B, Keene DR, Paulsson M, Koch M, Wagener R (2006) Collagen XXVIII, a novel von Willebrand factor A domain-containing protein with many imperfections in the collagenous domain. J Biol Chem 281:3494–3504

Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 92:827–839

Vuoristo S, Virtanen I, Takkunen M, Palgi J, Kikkawa Y, Rousselle P, Sekiguchi K, Tuuri T, Otonkoski T (2009) Laminin isoforms in human embryonic stem cells: synthesis, receptor usage and growth support. J Cell Mol Med 13:2622–2633

Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI, Goldberg VM (1994) Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Jt Surg Am Vol 76:579–592

Warden S, Zaleske DJ, Glowacki J (2004) Fate of a chimeric joint construct in an ectopic site in SCID mice. Cell Transplant 13:161–168

Weckroth M, Vaheri A, Lauharanta J, Sorsa T, Konttinen YT (1996) Matrix metalloproteinases, gelatinase and collagenase, in chronic leg ulcers. J Investig Dermatol 106:1119–1124

Wienke D, MacFadyen JR, Isacke CM (2003) Identification and characterization of the endocytic transmembrane glycoprotein Endo180 as a novel collagen receptor. Mol Biol Cell 14:3592–3604

Wu Y, Ip JE, Huang J, Zhang L, Matsushita K, Liew CC, Pratt RE, Dzau VJ (2006) Essential role of ICAM-1/CD18 in mediating EPC recruitment, angiogenesis, and repair to the infarcted myocardium. Circ Res 99:315–322

Wu M, Wu ZF, Merajver SD (2007) Rho proteins and cell-matrix interactions in cancer. Cells Tissues Organs 185:100–103

Xian X, Gopal S, Couchman JR (2010) Syndecans as receptors and organizers of the extracellular matrix. Cell Tissue Res 339:31–46

Xiang G, Schuster MD, Seki T, Kocher AA, Eshghi S, Boyle A, Itescu S (2004) Down-regulation of plasminogen activator inhibitor 1 expression promotes myocardial neovascularization by bone marrow progenitors. J Exp Med 200:1657–1666

Xiang G, Schuster MD, Seki T, Witkowski P, Eshghi S, Itescu S (2005) Downregulated expression of plasminogen activator inhibitor-1 augments myocardial neovascularization and reduces cardiomyocyte apoptosis after acute myocardial infarction. J Am Coll Cardiol 46:536–541

Yates KE, Allemann F, Glowacki J (2005) Phenotypic analysis of bovine chondrocytes cultured in 3D collagen sponges: effect of serum substitutes. Cell Tissue Bank 6:45–54

Yilmaz M, Christofori G (2009) EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev 28:15–33

Yozai K, Shikata K, Sasaki M, Tone A, Ohga S, Usui H, Okada S, Wada J, Nagase R, Ogawa D, Shikata Y, Makino H (2005) Methotrexate prevents renal injury in experimental diabetic rats via anti-inflammatory actions. J Am Soc Nephrol 16:3326–3338

Zhao S, Wehner R, Bornhäuser M, Wassmuth R, Bachmann M, Schmitz M (2010) Immunomodulatory properties of mesenchymal stromal cells and their therapeutic consequences for immune-mediated disorders. Stem Cells Dev 19:607–614

Zhou J, Ding M, Zhao Z, Reeders ST (1994) Complete primary structure of the sixth chain of human basement membrane collagen, alpha 6(IV). Isolation of the cDNAs for alpha 6(IV) and comparison with five other type IV collagen chains. J Biol Chem 269:13193–13199

Zimmermann WH, Didié M, Wasmeier GH, Nixdorff U, Hess A, Melnychenko I, Boy O, Neuhuber WL, Weyand M, Eschenhagen T (2002) Cardiac grafting of engineered heart tissue in syngenic rats. Circulation 106(12 Suppl 1):I151–I1157

Chapter 3
Stem Cell Niche

Chenhui Wang, Pei Wen, Pei Sun, and Rongwen Xi

Abstract The adult stem cells, or tissue-specific stem cells, are essential for maintaining tissue homeostasis and commonly reside in specific local microenvironment named niche. The niche keeps stem cells in multipotent/unipotent state and prevents them from precocious differentiation, and in some cases, aligns them and promotes asymmetric division to produce differentiated progenies for tissue regeneration. The niches employ a variety of factors including cell adhesion molecules, extra cellular matrix, growth factors and cytokines in a tissue-specific manner to regulate the resident stem cells. Stem cells in turn may also contribute to niche integrity and function. Continuous elucidation of stem cell niche regulation at the cellular and molecular level would help understanding tissue homeostasis and disease mechanisms, and may also provide useful strategies for therapeutic application of stem cells.

3.1 Introduction

Unlike embryonic stem cells, which possess the innate self-replicating capacity (Ying et al. 2008), the maintenance of most adult stem cells, if not all, requires stimuli from specialized local microenvironment, or niche. Dynamic interactions between niches and stem cells govern tissue homeostasis and repair under physiological and pathological conditions throughout life. Deregulation of the stem cell niches has been implicated in many diseases, including aging, cancer and degenerative diseases (Voog and Jones 2010).

The stem cell niche hypothesis was initially put forward by Schofield, who proposed that the maintenance of stem cells requires association with a complement

C. Wang • P. Wen • P. Sun, Ph.D. • R. Xi (✉)
Zhongguancun Life Science Park, National Institute of Biological Sciences,
7 Science Park Road, Beijing 102206, China
e-mail: xirongwen@nibs.ac.cn

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_3,

of cells, a 'niche' (Schofield 1978). However, it was not fully appreciated until studies in the model organisms, *Caenorhabditis elegans* and *Drosophila melanoganster*, demonstrated that the supporting stromal cells are important for the maintenance and self-renewal of germline stem cells. Subsequently, as new techniques and tools for charactering stem cells in vivo are accessible, the stem cell niches are accompanyingly identified and characterized in many mammalian tissues. Because stem cells are usually regulated by both cellular niche cells and non-cellular components, the stem cell niche is currently defined as the local tissue microenvironment that houses and maintains stem cells (Morrison and Spradling 2008).

Studies on both invertebrate and vertebrate stem cell niches in a variety of tissues revealed some principles of their functions. The stem niche controls stem cell self-renewal and prevent their precocious differentiation by secreting signaling molecules or cell-surface ligands, and anchors stem cells in place by utilizing cell adhesion molecules or the extracellular matrix. The niche also frequently positions stem cells in a way facilitating their asymmetric cell divisions, so that after each cell division, one daughter will remain aside the niche to continue self-renewing, while the other daughter will leave the niche and differentiate. Because of the intimate relationships between stem cells and their niches, mimicking the in vivo microenvironment could also help stem cell with in vitro expansion and functional integration into damaged tissues for future stem cell-based therapies. Thus, a comprehensive understanding of the molecular mechanism underlying the niche function not only contributes to our understanding of tissue homeostasis control and diseases, but also helps to put a step forward for the clinical application of stem cells.

Owing to advantages in simple tissue structure and availability of sophisticated genetic tools, studies in simple model organisms such as *Drosophila melanoganster* have pioneered our understanding of the niche, with clear demonstration of cellular composition and molecular basis of physical interaction and signaling regulation in the stem niches. Although adult stem cells in mammals are usually difficult to identify due to tissue complexity, with the identification of more reliable stem cell markers and endeavors of many researchers, tremendous progresses have also been made for adult stem cell niches in mammals. In the following parts, some examples of the best studied stem cell niches from invertebrates to mammals are introduced, with emphases on the structural composition and molecular functions. Subsequently we summarize the general features of the stem cell niche and discuss future challenges and clinical perspective on the stem cell niche.

3.2 *C. elegans* Germline Stem Cell Niche

The principle of cell-cell interaction in controlling stem cell behavior was first described in the worm gonad in early 1980s. In the *C. elegans* hermaphrodite gonad, there is one somatic cell at the distal end known as the distal tip cell (DTC). Germline stem cells (GSCs) are localized within the mitotic germ cell region close to the DTC tip (Fig. 3.1a). Moving along the distal-proximal axis, germ cells gradually switch

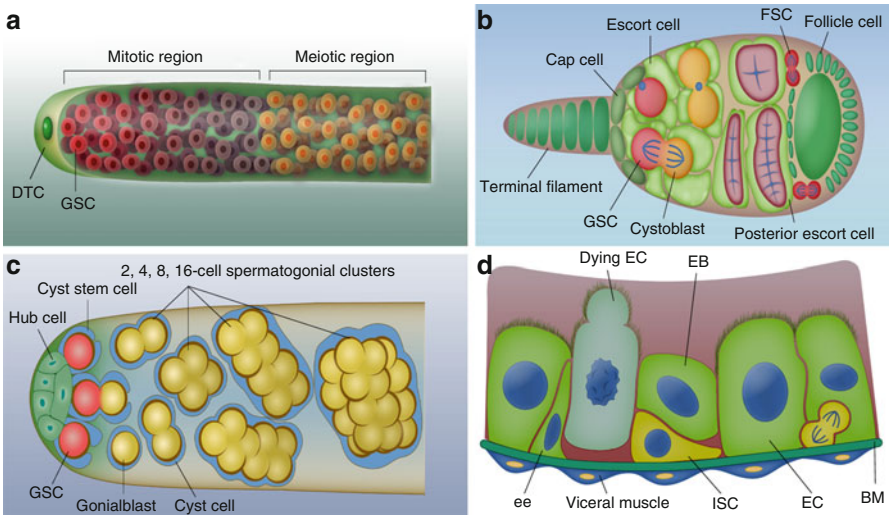


Fig. 3.1 The anatomy of *C. elegans* and *Drosophila* stem cell niches. (**a**) *C. elegans* germline stem cell (GSC) niche. GSCs are located in the mitotic region (*red*). The distal tip cell (DTC) (*green*) provides both physical support and signaling instructions to maintain GSCs. (**b**) *Drosophila* ovarian GSC and follicle stem cell (FSC) niches. Cap cells together with terminal filament and escort cells constitute the ovarian GSC niche. Cap cells anchor the GSCs by forming adherens junctions, and produce instructive signals to maintain GSCs. Daughter cells of GSCs positioned outside the GSC niche are differentiating cystoblasts. Two FSCs at the mid region of the germarium are responsible for the generation of the follicle cells that encapsulating the developing germline cysts. FSCs are in contact with the neighboring posterior escort cells and underlying basal lamina. (**c**) *Drosophila* male GSC niche. The male GSC niche is composed of hub cells and cyst stem cells. Similar with the ovarian counterparts, male GSC daughter cells positioned outside the niche become differentiating gonialblasts, which subsequently undergo four rounds of transit amplifying divisions with incomplete cytokinesis, generating 16-cell spermatogonial clusters. (**d**) *Drosophila* intestinal stem cell (ISC) niche. ISCs in the midgut are directly associated with a thin layer of basement membrane. The underlying visceral muscle secretes multiple signaling molecules to regulate ISC maintenance. The dying ECs may produce mitogens to stimulate ISC proliferation in response to various damage. *EB* enteroblast, *EC* enterocyte, *ee* enteroendocrine cell. Art works in this and subsequent figures are provided by Ning Yang

from mitosis to meiosis and subsequently develop into functional gametes (Kimble and Crittenden 2007). DTC is crucial for maintaining GSCs, because laser ablation of DTC causes GSC elimination, as GSCs are switched from mitosis to meiosis and subsequently differentiate. Also, when the location of male DTC was genetically manipulated, the axis of the gonad was disrupted and ectopic mitotic germ cells were formed around the mislocalized DTC (Kimble and White 1981). These data demonstrate that DTC is both necessary and sufficient for the maintenance of GSCs. Interestingly, the DTC sends short processes to encapsulate distal-most germ cells and long processes extending as many as 30 germ cells (Crittenden et al. 2006), which might provide a unique physical environment to support a pool of stem cells by a single niche cell.

The DTC controls GSC self-renewal via GLP-1/Notch signaling pathway (Crittenden et al. 1994; Henderson et al. 1994). The two DSL ligands LAG-2 and APX-1 are expressed in the DTC (Nadarajan et al. 2009), while the Notch-like receptor GLP-1 is expressed in germ cells in the mitotic region. Disruption of GLP-1/Notch signaling results in stem cell loss, whereas GLP-1 gain-of-function mutation leads to GSC overproliferation (Austin and Kimble 1987; Berry et al. 1997; Lambie and Kimble 1991). Activation of GLP-1/Notch signaling in GSC leads to the expression of downstream target fbf-2, which in turn represses the expression of differentiation-promoting genes including GLD-1, 2 and 3 (Byrd and Kimble 2009; Crittenden et al. 2002; Eckmann et al. 2004; Kimble and Crittenden 2007; Suh et al. 2009).

A body of knowledge has been acquired regarding the mechanisms regulating the DTC formation and maintenance. Briefly, the DTC is descended from somatic gonadal progenitor cell (SGP) through asymmetric division. The Wnt/β-catenin asymmetric (WβA) pathway plays central role in DTC specification. Activation of WβA pathway promotes the DTC fate through upregulating the expression of its direct target *ceh-22* (Lam et al. 2006). By contrast, NHR-25 represses the DTC fate by antagonizing WβA pathway (Asahina et al. 2006). In addition, the HLH-2/daughterless transcription factor is implicated in the DTC specification as well as maintenance (Chesney et al. 2009; Karp and Greenwald 2004). Of note, both WβA pathway and *ceh-22* are required and sufficient to specify the DTC fate. Loss of WβA pathway or *ceh-22* results in loss of the DTC, while over-activation of WβA pathway or *ceh-22* produces extra DTCs (Kidd et al. 2005; Lam et al. 2006; Siegfried et al. 2004; Siegfried and Kimble 2002).

3.3 Stem Cell Niches in *Drosophila*

3.3.1 Germline Stem Cell Niche in the Drosophila Ovary

The anatomic structure of the *Drosophila* gonad is well defined. The female and male GSCs can be reliably identified in vivo by their localization and by specific cellular markers, and remain accessible to sophisticated genetic manipulations. Consequently, they serve as excellent model systems to study niche regulation of stem cells. In fact, the molecular mechanisms of *Drosophila* GSC-niche regulation are among the best studied and have provided a conceptual framework for the niche study in mammalian systems.

In the Drosophila ovary, GSCs can be identified by their anterior-most location in the germarium and the presence of a unique organelle named spectrosome. In each germarium, five to ten terminal filament (TF) cells, four to six cap cells and GSC-contacting escort cells constitute the female GSC niche that houses two or three GSCs (Fig. 3.1b). Normally, GSCs undergo asymmetric divisions. Upon each division, one daughter remains within the niche and adopts the GSC fate, while the other daughter is positioned outside the niche and invariably differentiates into a cystoblast (CB), which will commit four rounds of incomplete mitosis to generate a 16-cell cyst and ultimately a new oocyte.

The cap cells are the principal component of the GSC niche (Xie and Spradling 2000), which anchor GSCs by forming DE-cadherin-mediated adherens junctions between the GSCs and the cap cells (Song et al. 2002). Loss of this adhesion would cause GSCs to leave their niche and differentiate. In addition to the role in physical support, the cap cells also provide signals that are essential for GSC maintenance. They secrete BMP family ligands Dpp and Gbb, which locally activate receptors on GSCs and suppress the expression of a differentiation-promoting gene, *bag of marbles* (*bam*). In cystoblasts, the BMP signaling activity diminishes, which results in the release of *bam* repression and the initiation of differentiation. BMP signaling is required for GSC maintenance, as compromised BMP signaling pathway transduction in GSCs causes their precocious differentiation. Dpp overexpression is also sufficient to stimulate GSC self-renewal and block GSC differentiation, leading to the accumulation of GSC-like cells in the ovariole (Chen and McKearin 2003; Song et al. 2004; Xie and Spradling 1998). GSC-contacting escort cells are also an important component of GSC niche, as blockade of JAK/STAT signaling in escort cells results in loss of GSCs (Decotto and Spradling 2005). In addition, unpaired (Upd) produced from TF cells activates JAK/STAT signaling in cap cells and escort cells, leading to augmented expression of Dpp (Lopez-Onieva et al. 2008; Wang et al. 2008). Therefore, TF cells also contribute to the niche.

Much progress has been made in understanding how niche controlled BMP signaling activity is restricted to GSCs. That has been reviewed somewhere else (Chen et al. 2011; Losick et al. 2011). Briefly, JAK/STAT signaling seems to be necessary and sufficient for dpp expression in cap cells, while Lsd1 inhibits dpp expression in escort cells, as knockdown of Lsd1 in escort cells augments dpp transcription (Eliazer et al. 2011). In addition, the heparin sulfate glycoprotein Dally, and the type IV collagen Viking are required to restrict diffusion of Dpp outside the niche (Guo and Wang 2009; Hayashi et al. 2009; Wang et al. 2008). Moreover, the serine/threonine kinase Fused, together with the E3 ligase Smurf direct the degradation of BMP receptor Thickvein (Tkv) in CBs, allowing for CB differentiation (Xia et al. 2010).

The niche function also requires Yb and Piwi, which are required in the somatic niche cells to maintain GSCs (Cox et al. 1998; King and Lin 1999). GSCs also send signals to the niche to regulate niche function. Delta, the ligand for the Notch pathway, is specifically expressed in the germ cells, and activates Notch in the niche cells for their specification during the development for their maintenance during adulthood (Song et al. 2007; Ward et al. 2006).

3.3.2 Follicle Stem Cell Niche in the Drosophila Ovary

In each germarium, two follicle stem cells (FSCs), which generate follicle cells to envelop the developing germ cells, are located near the boundary between the 2A and 2B regions (Nystul and Spradling 2007) (Fig. 3.1b). So far there is no reliable cellular marker to identify FSCs. It has been suggested multiple signal molecules produced from the TF/cap cells, including Hedgehog (Hh), Wingless (Wg) and Dpp, are all required for the long-term maintenance of FSCs, indicating that these

signaling pathways function cooperatively to regulate FSC behavior (Forbes et al. 1996; Kirilly et al. 2005; Song and Xie 2003; Zhang and Kalderon 2001). Therefore, the GSC niche also functions as a part of the niche for FSCs.

Apart from that, FSC-contacting posterior escort cells located near the region 2A/2B border could be an essential component of the FSC niche as well. Escort cells do not turn over regularly and do not move along with cysts at the junction of 2A and 2B region (Morris and Spradling 2011). In addition, E-cad and Armadillo/β-catenin enriched at the junctions between FSCs and its adjacent cells are required for the maintenance of FSCs (Song and Xie 2002), suggesting adherens junctions anchor FSCs to the escort niche cell. Besides, integrin-mediated FSC anchoring to the basal lamina is also required for the long-term maintenance of FSCs (O'Reilly et al. 2008), suggesting that extracellular matrix is a critical component of the FSC niche.

Although it is poorly understood how these extrinsic niche signals act on FSCs to regulate their self-renewal, some intrinsic factors have been identified to be involved in this process. The ATP-dependent remodeling factor Domino (DOM) is required for FSC self-renewal (Xi and Xie 2005), while two polycomb genes Psc and Su(z)2 function redundantly and necessarily in FSCs for their differentiation. Loss of Psc and Su(z)2 ultimately leads to neoplastic tumor (Li et al. 2010). Further studies would provide more profound insights into the fundamental yet intricate mechanisms by which the niche signals link to intrinsic factors for the control of FSC self-renewal.

3.3.3 Germline Stem Cell Niche in the Drosophila Testis

The male GSC niche is also well-studied in Drosophila. A cluster of somatic cells (which form a hub) are located at the anterior tip of the testis and serve as the niche for both GSCs and the cyst stem cells (CySCs, or cyst progenitor cells) (Fig. 3.1c). About 8–10 GSCs reside around each hub, and each GSC is encapsulated by two CySCs. After each asymmetric division, the GSC produces a new GSC that remains in contact with the hub and a differentiating daughter namely gonialblast, which is positioned outside the niche and subsequently undergoes four rounds of transit amplifying divisions with incomplete cytokinesis, generating a 16-cell spermatogonial cluster. Spermatogonia further differentiate into spermatocytes which undergo meiosis and ultimately produce sperms. GSCs and gonialblasts contain a spectrosome as their counterparts in the ovary, while differentiated germ cell clusters have a branched fusome. The CySC divides coordinately with GSC division to produce a pair of cyst cells which enclose the differentiating gonialblast.

The activation of JAK/STAT signaling by the hub cells secreted ligand Upd was initially suggested to be necessary and sufficient for both GSCs and CySCs self-renewal (Kiger et al. 2001; Tulina and Matunis 2001). However, intrinsic activation of JAK/STAT signaling pathway in GSC alone stimulates the expression of DE-cadherin, which mediates GSC adhesion to hub cells, but is not sufficient to promote GSC self-renewal (Leatherman and Dinardo 2010). It turns out that activation of JAK/STAT signaling in CySCs induces the expression of zfh-1, which

stimulates the expression of BMP ligands Dpp and Gbb. BMP signaling activation in GSCs represses the transcription of differentiation-promoting factor *bam* and ultimately leads to GSCs self-renewal away from the hub cells (Kawase et al. 2004; Leatherman and Dinardo 2008). Therefore, in addition to the hub, CySCs may also be important components of the male GSC niche.

Like the ovarian counterpart cap cells, hub cells also express BMP ligand Gbb and Dpp. In addition, the male GSC niche also utilizes ECM to restrict BMP ligands diffusion. Dally-like instead of Dally is involved in this process (Hayashi et al. 2009).

The hub is derived from somatic gonadal precursors (SGPs) in the embryonic gonad. Notch and EGFR signaling have been implicated in hub cell specification. Notch signaling promotes hub specification, while EGFR signaling acts antagonistically with Notch to suppress hub differentiation (Kitadate and Kobayashi 2010). Interestingly, CySCs shares a common precursor with hub cells and can contribute to hub replenishment under certain circumstances, highlighting the dynamic nature of stem cell-niche relationship (Dinardo et al. 2011; Voog et al. 2008).

Studies in the male GSC niche also provide insights into the mechanisms of spindle orientation for asymmetric division of stem cells. The centrosome is replicated during interphase, and during mitosis, the mitotic spindle is mostly perpendicular to the hub-GSC interface. DE-cadherins could act through membrane-bound β-catenin and adenomatous polyposis coli (APC) to anchor the spindle pole (Yamashita et al. 2003). Interestingly, the mother and daughter centrosomes are asymmetrically inherited after mitosis by the two daughters of one stem cell, as the mother centrosome is always inherited by the daughter retaining stem cell fate (Yamashita et al. 2007).

3.3.4 Intestinal Stem Cell Niche in the Drosophila Midgut

The *Drosophila* gastrointestinal tract shows a high similarity to the mammalian intestine in development, cell composition and physiological function. In addition, the *Drosophila* intestinal epithelium is also maintained by multipotent intestinal stem cells (ISCs) (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). The epithelium is composed of a layer of cells projecting to the gut lumen, with highly organized apical-basal polarity. The ISCs, the only epithelial cells that are competent to undergo mitosis, reside at the basal surface of the epithelium and directly contact with the basement membrane (BM) composed of ECM, which separates the gut epithelium with the surrounding visceral muscles. An ISC undergoes asymmetric division to produce two daughters with one retaining ISC fate and the other undergoing differentiation. The differentiated daughter, named enteroblast (EB) will differentiate into either an absorptive enterocyte (EC) or a secretory enteroendocrine (ee) cell (Fig. 3.1d). Notch signaling plays a critical role in the cell fate determination of intestinal cell linage (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). ISCs specifically express a Notch ligand Delta (Dl), which actives Notch in the EBs and promotes them to differentiate into ECs or ee cells. The expression level of Dl in the ISCs is variable from one ISC to another. It is believed

that the high Dl level activates Notch at a high level in EB to promote its differentiation towards EC fate, whereas the low Dl level activates Notch at a low level to allow EB to differentiate toward ee fate (Ohlstein and Spradling 2007).

ISCs do not directly contact with any fixed stromal cells. The underlying visceral muscle is proposed to be a major component of the ISC niche. Wingless is the first identified molecule produced by the niche, which is able to traverse through the BM and activates the canonical Wnt signaling pathway in ISCs to regulate their long-term maintenance and proliferation (Lin et al. 2008). The visceral muscle also expresses Unpaired (Lin et al. 2009), the ligand of JAK/STAT pathway, and Vein (Biteau and Jasper 2011; Buchon et al. 2010; Jiang et al. 2010; Xu et al. 2011), the ligand for EGFR, which respectively activate JAK/STAT and EGFR/Ras signaling in ISCs to regulate ISC maintenance and proliferation. Recently, the Drosophila insulin-like peptides, dILP3, was found to be produced by the visceral muscle cells as well, which activates ISCs and expands ISC population to promote adaptive growth of intestine in response to nutrition availability (O'Brien et al. 2011). It is noteworthy that activation of any one of Wingless, JAK/STAT or EGFR signaling pathway alone in ISCs is not sufficient to completely block ISC differentiation (Lee et al. 2009; Lin et al. 2009; Xu et al. 2011). Therefore, the self-renewal of ISCs is likely controlled by a cooperative action of multiple signaling pathways. Several JAK/STAT and EGFR ligands, such as Upd3, Spitz and Karen, could also be detected in epithelial cells, including ISCs, progenitor cells and ECs (Beebe et al. 2009; Biteau and Jasper 2011; Jiang et al. 2009, 2010; Lin et al. 2009; Liu et al. 2010; Xu et al. 2011), especially under stress conditions (Buchon et al. 2009, 2010; Jiang et al. 2010), suggesting that non-stem cells in the intestinal epithelium could also contribute to niche function. The diverse and dynamic expression of those maintenance signals suggest that the niche function can be dynamically regulated in co-ordinance with environmental changes.

3.4 Stem Cell Niches in Mammals

Increasing evidence suggests that adult stem cells in mammals are also housed and maintained by the niches, although most of the tissue-specific stem cell niches have not been rigorously verified largely due to their associated tissue complexity. In addition to the common scenarios regarding the functional relationships between the stem cells and the stem cell niches, there could be distinct mechanisms uniquely exploited in mammalian stem cells but not stem cells in invertebrate. For example, the invertebrate stem cells are usually mitotically active. In contrast, the mammalian adult stem cells are often in a relatively quiescent state. In many cases, there seems to be two populations of stem cells with distinct niche locations: quiescent and active stem cells. In the following parts, some examples of the best studied mammalian stem cells and their associated niches are described and discussed, focusing on the physical composition and signaling interactions within the stem cell niches.

3.4.1 The Hematopoietic Stem Cell Niche

As mentioned before, the niche hypothesis was first proposed based on studies on the rodent hematopoietic stem cell (HSC) system several decades ago, although the exact location of the HSCs in the bone marrow (BM) had been a mystery. Until recent years, considerable progresses have been made to understand the HSC niche in the BM. The current view is that there are two HSC niches within the BM, the osteoblastic niche on the endosteal surface and the vascular niche of sinusoid endothelial cells (Fig. 3.2a).

3.4.1.1 The Osteoblastic Niche

Before the in vivo HSC niche was characterized, a series of in vitro studies showed that osteoblastic cell lines were capable of supporting primitive hematopoietic cells for a long term in ex vivo culture systems (Taichman et al. 1996). These observations provided an important hint for finding the HSCs niche in the BM. Osteoblastic cells were first demonstrated to participate in HSC regulation in vivo by two simultaneous studies working with different engineered mouse models (Calvi 2003; Zhang 2003). Both cases of genetic manipulation of the mouse models induced an increase in the number of osteoblasts and trabecular bone, and the number of HSCs increased accompanyingly. Consistently, ablation of osteoblasts by expression thymidine kinase specific in the osteoblasts leads to a decrease of primitive hematopoietic cells in the BM and an increase of extrameduallary hematopoiesis (Visnjic et al. 2004). It is noteworthy that only N-cadherin+ osteoblasts are associated with HSCs (Zhang 2003). However, N-cadherin is not required for HSC maintenance as loss of N-cadherin does not lead to HSC depletion or defective hematopoiesis (Kiel et al. 2009).

There are additional molecules produced by the osteoblasts that have been implicated in the regulation of HSCs, such as Angiopoietin-1, Thrombopoietin, Osteopontin (Opn), and CXCL12 (also called SDF-1). Angiopoietin-1 and Thrombopoietin interact with their receptors (Tie-2 and MP1 respectively) expressed on the HSCs to maintain HSC quiescence (Arai et al. 2004; Yoshihara et al. 2007). Opn, a glycoprotein, negatively regulate HSC proliferation and the size of the HSC pool, perhaps via interaction with integrins and CD44 (Nilsson et al. 2005; Stier et al. 2005). CXCL12, a chemokine that activates the receptor CXCR4 in HSCs, is also important for HSC quiescence and maintenance in the BM (Nie et al. 2008; Sugiyama et al. 2006a). CXCL12 is also expressed in other non-osteoblast cells, including endothelial cells, and a subset of reticular cells scattered in the BM. Thus, these cells may also play a role in the BM niche (Sugiyama et al. 2006a). The Wnt signaling may also regulate HSC quiescence, as osteoblast-specific overexpression of the canonical Wnt inhibitor Dikkopf1 (Dkk1) results in HSC activation (Fleming et al. 2008), although the requirement of Wnt signaling has not been directly demonstrated.

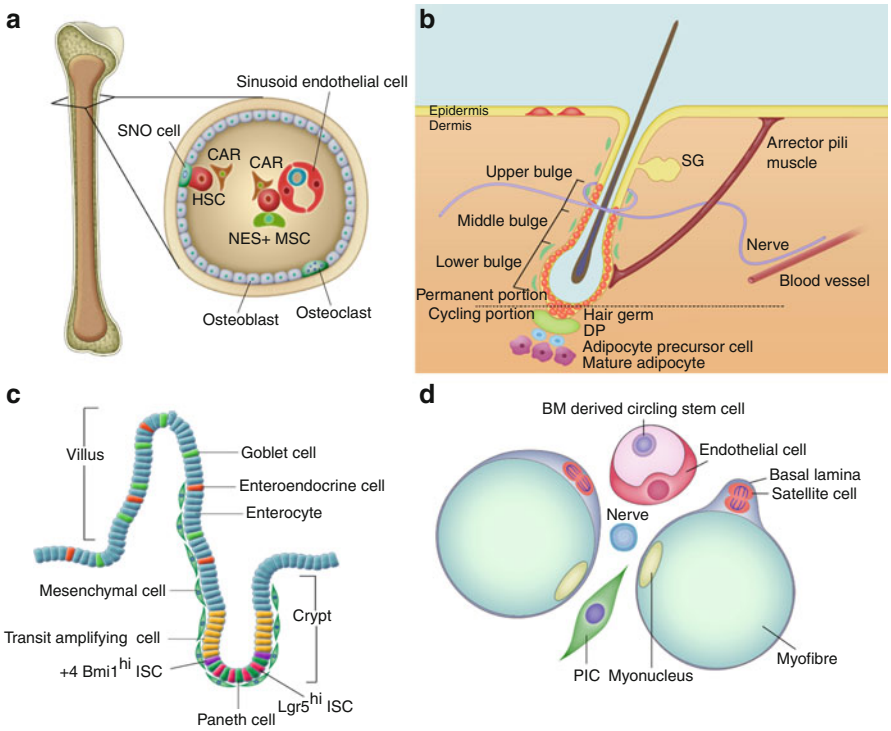


Fig. 3.2 The anatomy of mammalian stem cell niches. (**a**) Hematopoietic stem cell (HSC) niche. HSCs in the bone marrow reside in two niche locations: at the endosteal surface associate with spindle-shaped N-cadherin^{+}CD45^{-} osteoblastic (SNO) cells, and at the microvasculature associated with sinusoid endothelial cells and mesenchymal stem cells (MSCs) expressing Nestin. HSCs at both regions are frequently associated with CXCL12-abundent reticular (CAR) cells. (**b**) Stem cell niches in skin. A diagram of hair follicle (HF) in telogen. In the epidermis, stem/progenitor cells are located in the basal layer and differentiate into suprabasal cells. The basement membrane separates basal layer from the underlying dermis. The HFSCs reside in the bulge region below the sebaceous gland (SG). The mesenchymal dermal papilla (DP) and adipocyte lineages are crucial for follicle stem cells maintenance and activation. The upper bulge is wrapped by sensory nerve fibers, which release Sonic hedgehog (Shh) to induce Gli1 expression in adjacent upper stem cells. The activation of Hh pathway is essential for the upper stem cells to gain the potential to become epidermal stem cells during wound healing. (**c**) Intestinal stem cell (ISC) niche in the small intestine. Bmi1^{hi} ISCs are located at the +4 position from the crypt bottom and contact with paneth cells and transit amplifying cells. Lgr5^{hi} ISCs are located at the crypt bottom and surrounded by paneth cells which form the niche for Lgr5^{hi} ISCs. A hierarchy between Bmi1^{hi} ISCs and Lgr5^{hi} ISCs has been suggested recently. (**d**) Muscle stem cell niche. Two types of muscle-resident stem cells have been described. Satellite cells are located beneath the basal lamina and are in contact with myofibers. They could undergo planar symmetric divisions and apical-basal asymmetric divisions. The recently identified muscle stem cells – PW1+Pax7- interstitial cells (PICs) are located between myofibers. Both PICs and bone marrow-derived cells are able to generate functional satellite cells during regeneration

3.4.1.2 The Vascular Niche

Increasing evidence indicates that the vasculature in the BM may also serve as the HSC niche. Multiple cell types have been reported to make up the HSC vascular niche. A simple combination of three SLAM family receptors is found to be able to specifically distinguish the stem and progenitor cells and thus make it possible to detect the HSC niche in tissue section (Kiel et al. 2005). With the help of these new markers, many of the hematopoietic stem/progenitor cells (HSPCs) were found to be mainly located in the perivascular region. Consistently, an in vivo imaging study revealed that after transplantation, the labeled primitive hematopoietic cells could home to SDF-1–rich subdomains of microvessels in the bone marrow, where they persisted and increased in number over time (Sipkins et al. 2005). These studies suggest the perivascular region could serve as the HSC niche. VEGFR2 and VEGFR3 are expressed in sinusoidal endothelial cells (SECs), but not smooth-muscle-invested arterioles or osteoblasts. VEGFR2 is not required for normal HSC homeostasis. However, upon severe myelosuppressive damage, VEGFR2-mediated SEC regeneration is critical for HSC engraftment and reconstitution (Hooper et al. 2009).

Recently, a population of nestin-expressing (NES+) mesenchymal stem cells (MSCs), which are exclusively distributed in perivascular region, has been identified to act as an unique niche of bone marrow HSC. NES+ cells are physically associated with HSCs and express multiple HSC maintenance genes including CXCL12 and Angiopoietin-1. In vivo ablation of NES+ MSC cells leads to significant reduction of long term HSCs (LT-HSCs) number (Mendez-Ferrer et al. 2010).

Additionally, CXCL12-abundant reticular (CAR) cells are the major source of CXCL12. And most HSCs near endosteum or the sinusoidal endothelium, if not all, are in contact with CAR cells (Sugiyama et al. 2006b). Selective ablation of CAR cells cause reduction of HSCs number by approximately 50% and HSCs become more quiescent, suggestive of CAR cells as an essential HSC niche component (Omatsu et al. 2010). Both CAR cells and NES+ MSCs are competent to differentiate into adipocytes and osteoblasts, suggesting that there may be some overlap between these two cell types.

Therefore, the HSC pool in the BM could be divided into two subpopulations or states: the quiescent population, which is inactive and functions as a potent reservoir for the long-term maintenance of HSCs, and the active population, which is highly proliferative and responsible for the daily regeneration. The HSCs in the osteoblastic niche are BrdU retaining cells, and the signals from the osteoblastic niche usually regulate the quiescence of the HSCs. In contrast, the majority of HSCs identified by the SLAM markers are mitotically active (Kiel et al. 2005). These observations lead to a proposal that the osteoblastic niche and the vascular niche could function to support quiescent (reserved) and activated HSCs, respectively (Zhang and Li 2008).

3.4.2 Skin Stem Cell Niche

The mammalian skin, which is under constant turnover, serves as a physical barrier to protect the body from many environmental stresses such as bacteria infection, dehydration and UV-irradiation. The epidermis appendages such as hair follicles, nail, oil and sweat glands endow additional sophisticated functions to the body. The epidermis is comprised of stratified layers of progenitors and differentiated cells, and the stem cells or progenitors are believed to reside in the basal layer above the dermis (Fuchs 2009; Watt 1998) (Fig. 3.2b). Attached to the BM that separates epidermis from dermis, the basal cells can undergo asymmetric division to generate suprabasal spinous cells, which subsequently move upward and became enucleated and finally shed from the body. Notch signaling, p63 and microRNAs are important for the basal-to-suprabasal switch of the progenitor cell (Blanpain and Fuchs 2006; Moriyama et al. 2008; Yi et al. 2008).

The skin with hair can be divided into the following structural units: each with a hair follicle (HF), sebaceous gland (SG) and interfollicular epidermis (IFE). Sequentially down from the SG is the bulge where stem cells reside, outer root sheath, inner root sheath, hair shaft, transit amplifying matrix cells that envelop a group of mesenchymal cells, and dermal papilla (DP) (Fig. 3.2b). The adult HF constantly undergoes rounds of degeneration (catagen), rest (telogen) and growth (anagen), known as hair cycle. HF stem cells (HFSCs) provide the source of proliferation during anagen. In the destructive catagen phase, the matrix cells undergo programmed cell death and bring up the DP to the position that is underneath the (secondary) hair germ, the early progenies of bulge stem cells. The DP plays an inductive role in maintaining HFSCs in quiescent state and competent for the next cycle of growth (Blanpain and Fuchs 2006) (Fig. 3.2b). Normally, HFSCs do not contribute to the maintenance of SG and IFE. However, during the repairing process after wounding, they can regenerate the damaged epidermis and SG. HFSCs can be divided into two populations based on their location with the basal lamina: basal and supra-basal populations. These cells differ in their expression signatures, but both populations are able to self-renew in vitro and share the same differentiation potential (Blanpain et al. 2004).

The epithelial-mesenchymal interactions are important to regulate HFSCs (Blanpain and Fuchs 2009). Among the signaling pathways, Wnt and BMP are the most intensively studied. From embryonic HF initiation to adult stem cell self-renewal and differentiation, Wnt signaling plays multiple important roles during these processes. Loss of β-catenin, which complexes with TCF/LEF transcription factors to activate Wnt-response genes, completely blocks HF formation, while overexpression of an activated form leads to de novo HF morphogenesis (Gat et al. 1998; Huelsken et al. 2001). Elegant genetic and mathematical modeling show that Wnt ligands and the inhibitor Dkks pattern the HF spacing by a reaction–diffusion mechanism (Sick et al. 2006). In adult HF, β-catenin nuclear accumulation correlates with the transition from telogen to anagen, indicating the important roles of Wnt signaling in regulating stem cell self-renewal (Lowry et al. 2005). Wnt/beta-catenin signaling activities are also detected during matrix cell differentiation towards hair shaft (DasGupta and Fuchs 1999), and LEF1 rather than TCF3 in the bulge are required

for matrix cell differentiation. Despite these prominent roles, the source of Wnt ligands is difficult to probe, as there are dozens of Wnts in mammals with some expressed in the epithelium, yet others in the mesenchyme (Reddy et al. 2001). The BMP pathway has long been known for its inhibitory effects on HF morphogenesis and adult HFSC proliferation (Blessing et al. 1993; Botchkarev et al. 1999). The mesenchyme produces a balanced level of BMP ligands and the antagonist noggin (Blanpain and Fuchs 2009). In activating the BMP receptor BMPR1a in HF epithelium leads to enhanced cycling of HFSCs and impaired differentiation (Kobielak et al. 2007). Other signaling pathways such as hedgehog and Notch are also involved in either regulating HF proliferation or differentiation (Blanpain and Fuchs 2009).

Recently, it has been found that sensory nerves regulate stem cell function in the upper bulge by producing Sonic hedgehog (Shh), which induces expression of Gli1 expression in adjacent stem cells. Gli1+ cells have the potential to become epidermal stem cells during wound healing. And the activity of these cells depends on Shh released from the perineural niche (Brownell et al. 2011). It is also worth additional attention that adipocyte precursor cells positively regulate follicle stem cell activity by producing platelet-derived growth factors (PDGFs). Lack of adipocyte precursor cells due to the inhibition of adipogenesis at early developmental phase in Efb1 knockout mice leads to defects in stem cell activation. And injection of WT adipocyte precursor cells into $Efb1^{-/-}$ skin at P21 is able to activate stem cell and rescue the hair cycling defects. A recent study further demonstrate that adipocyte precursor cells are sufficient to activate follicle stem cells (Festa et al. 2011).

3.4.3 Intestinal Stem Cell Niche

The mammalian intestinal epithelium turns over every 3–5 days, making it one of the most rapid self-renewing tissues in adult. In the small intestine of mouse, the gut epithelium is organized into numerous crypt/villi units, with the invaginations known as crypts and protrusions termed villi, surrounded by pericryptal fibroblasts and mesenchyme. The intestinal stem cells (ISCs) reside in the crypt and give rise to transit amplifying cells, which move upward and differentiate into absorptive enterocytes, mucos-secreting goblet cells and hormone-secretive enteroendocrine cells in the villi. Upon reaching the tip of villi, these cells undergo programmed cell death before shedding into the lumen. The ISCs also generate bactericidal Paneth cells, which are located in the bottom of the crypt (van der Flier and Clevers 2009) (Fig. 3.2c).

Two populations of stem cells have been identified with compelling evidence. Conventional long-term BrdU label retaining assay based on the "immortal strand" hypothesis suggests that ISCs are located just above the paneth cells at the +4 position from the crypt bottom. The polycomb group gene *Bmi1* is found to be specifically expressed in the cells located at the +4 position. Genetic lineage tracing mediated by Bmi1-CreER demonstrates that the *Bmi*-1 expressing cells can populate the whole epithelium 12 months after tamoxifen induction, further supporting that the $Bmi1^{+}$ cells at the +4 position behave as intestinal stem cells (Sangiorgi and Capecchi 2008). +4 position ISCs can be marked by mouse telomerase reverse transcriptase

(*mTert*)-GFP as well. Similar lineage tracing mediated by *mTert* -CreER further confirms that cells at +4 position give rise to all differentiated intestinal cell types (Breault 2008; Montgomery 2011).

Similar genetic tracing studies done by the Clevers group identify the crypt base columnar (CBC) cells which express a Wnt target gene Lgr5 and are interspersed among the paneth cells as bona fide ISCs. The Lgr5-expressing cells can regenerate the vili-crypt unit within 2 months after induction (Barker 2007). Interestingly, a single isolated $Lgr5^+$ stem cell could regenerate the intact crypt-villus organoid in vitro without the long postulated mesenchymal niche, suggesting that ISCs have an innate and robust self-organizing ability to direct the formation of a functional epithelium (Sato 2009). The identification of CBC as intestinal stem cells is further sustained by lineage tracing studies conducted with *Prominin 1* (Zhu 2009). Most recently, Clevers and colleges have shown that paneth cells constitute the niche for $Lgr5^+$ stem cells. Co-culture of sorted $Lgr5^+$ cells with paneth cells significantly promote the crypt-villus organoid formation. Additionally, selective ablation of paneth cells in vivo leads to loss of $Lgr5^+$ stem cells coincidently (Sato et al. 2011). Notably, $Lgr5^+$ stem cells divide symmetrically in their niche. They undergo "neutral competition" for niche occupation and the loser is expelled from the niche to undergo differentiation (Lopez-Garcia et al. 2010; Snippert et al. 2010).

Until most recently, the relationship between +4 position ISCs and $Lgr5^+$ ISCs was unclear. Interestingly, mTert-expressing ISCs have been reported to be able to give rise to $Lgr5^+$ ISCs, suggestive of a hierarchy between the slow-cycling and fast-cycling ISCs (Montgomery 2011). However, the $Lgr5^+$ ISCs also display significant telomerase activity (Schepers et al. 2011). Therefore it requires reconsideration whether mTert-expressing ISCs overlap with $Lgr5^+$ ISCs. Interestingly, a recent study shows that complete loss of $Lgr5^+$ ISCs by genetic ablation does not perturb the architecture and homeostasis of the intestinal epithelium, suggesting other stem cell pools can compensate for the loss of $Lgr5^+$ ISCs. Lineage tracing studies suggest that $Bmi1^+$ ISCs can replenish the fast-cycling $Lgr5^+$ ISCs both under normal condition and after injury (Tian et al. 2011), further supporting the existence of slow-cycling and fast-cycling ISCs, which can be marked by Bmi1 and Lgr5, respectively.

Multiple signaling pathways participate in the regulation of the gut homeostasis, including Wnt, BMP, Notch, Hedgehog, EphB and Ras pathways, and each of them have different roles in regulating cell proliferation, differentiation and migration. The Wnt/β-catenin pathway is the major pathway controlling ISC maintenance and self-renewal. High levels of nuclear β-catenin are found in the epithelial cells at the crypt bottom, but not in the epithelial cells in the villus. Disrupting Wnt pathway activity causes crypt loss, indicating that Wnt signaling is essential for ISC maintenance (Korinek et al. 1998). On the other hand, Wnt pathway activation by the loss of APC, a negative regulator of Wnt signaling, produces giant crypts because of hyperproliferation of intestinal progenitor cells (Andreu et al. 2005; Sansom et al. 2004). The source of the active Wnt ligand remains elusive. In situ results show that several Wnts are expressed in the crypt bottom, while several other Wnts are expressed in the mesenchymal cells (Girgenrath et al. 2006). BMP signaling

activated by the BMP ligands produced from the mesenchymal cells functions to restrict ISC proliferation and facilitate differentiation, as loss of Bmpr1a or expression of noggin inhibitor in intestine epithelium leads to intestinal polyposis (Haramis et al. 2004; He et al. 2004). Hedgehog signaling inhibits ISC proliferation and promotes their differentiation by inducing the expression of BMP ligands in the mesenchymal cells (Madison et al. 2005; van den Brink et al. 2004). These observations also indicate that the mesenchyme beneath the crypt has important role in regulating ISC behavior and could be an important constitute of the ISC niche.

3.4.4 Muscle Stem Cell Niche

Satellite cells, the best understood muscle-resident stem cells, are believed to be crucial for postnatal skeletal muscle growth and regeneration after injury. They are located between the plasma membrane of muscle fiber and basement membrane surrounding the muscle fiber (Fig. 3.1d). After injury, satellite cells are activated to generate myogenic precursor cells, which undergo transit amplification and differentiation before finally fuse to form multinucleated myofibers. Recent studies demonstrate that satellite cells are heterogeneous populations consisting of slow-cycling stem cells and fast-cycling progenitor cells. Both stem cells and progenitor cells express Pax7, but only progenitor cells express Myf5. $Pax7^{+}$ $Myf5^{-}$ satellite cells can undergo planar division (usually symmetric) and apical-basal division (usually asymmetric). There is a strong correlation between the fate and location of their daughter cells upon division. The daughter cell attached to basement membrane remains a self-renewing stem cell, and the other daughter positioned away from basement membrane becomes a committed myogenic cell (Kuang et al. 2007).

The host muscle fiber, extracellular matrix, microvasculature and interstitial cells constitute the niche for satellite cells (Kuang et al. 2008). Mice lacking the ECM component Laminin-α2 show defects in muscle growth and regeneration (Miyagoe et al. 1997), indicating a critical role of ECM in satellite cell function. Injured muscles could release HGF to activate the quiescent satellite cells, and the macrophage could release the TNF ligand TWEAK to promote muscle progenitors regeneration (Girgenrath et al. 2006; Tatsumi et al. 1998). Other growth factors and cytokines such as bFGF, IGF, BNDF, VEGF, PDGF, IL-6 and LIF could also regulate satellite cell proliferation and differentiation (Kuang et al. 2008). The Delta/Notch signaling pathway plays an important role for maintaining muscle stem cells (Conboy and Rando 2002). The ligand Delta-1 enriched in $Pax7^{+}Myf5^{+}$ progenitor cell is assumed to activate Notch signaling to promote self-renewal of the adjoining $Pax7^{+}Myf5^{-}$ stem cell. Blockage of Notch signaling leads to reduced stem cell self-renewal and regeneration ability (Conboy et al. 2003; Kuang et al. 2007). Intriguingly, crosstalk between Wnt and Notch signaling via GSK3β has been shown to be involved in the cell fate choices of activated satellite cells.

Over-activation of Wnt signaling pathway leads to premature muscle differentiation while its inactivation prevents muscle differentiation. The defects in muscle differentiation caused by enhancement of Notch signaling can be rescued by enhancement of Wnt signaling (Brack et al. 2008).

Emerging evidence suggest that non-satellite cells may contribute to myogenesis in response to injury. Transplanted adult bone marrow-derived cells (BMDC) can be converted to functional satellite cells following irradiation-induced damage (LaBarge and Blau 2002). Recently, a population of $PW1^{+}Pax7^{-}$ interstitial cells (PICs) have been identified to be able to generate satellite cells during regeneration, suggesting a hierarchy between these two muscle stem cell populations (Mitchell et al. 2010). The potential niche for PICs remains to be defined.

3.5 Key Components of the Stem Cell Niche

As described above, niche structure varies greatly from tissues to tissues and in different organisms. In terms of physical composition, some niches are relatively simple, composed of a single type of stromal cell, but some are rather complex, composed of multiple types of stromal cells and also non-cellular components. In terms of the stem cell types they host, some niches specifically host a single type of stem cells, and some rather simultaneously control more than one type of stem cells. However, all of these relatively well-characterized niches share certain common components, which are summarized as the following.

1. Physical support. The residence of stem cells within specific anatomic locations requires particular physical support including association with supportive stromal cells or basement membrane or both. The physical support keeps stem cells from being exposed to detrimental environment and prevents them from undergoing precocious differentiation. On the basis of physical association between stem cells and niches, two general types of niche -stromal niches and epithelial niches have been proposed (see below) (Morrison and Spradling 2008).
2. Secreted signals. The stromal cells in the niche commonly produce secreted signal molecules to directly regulate stem cell maintenance and self-renewal. Some niches require one principal signal for this function, whereas some niches require the cooperative function of multiple signals. These signaling activities often function to prevent the initiation of differentiation programs, thereby keeping stem cells in the undifferentiated states. The niche signaling also frequently regulates stem cell activity by promoting or inhibiting their division, therefore controls stem cell quiescence and activation.
3. Cell adhesion molecules. Stem cells commonly produce cell adhesion molecules for their anchorage to the niche. Cadherin-mediated cell-to-cell adhesion between the stem cells and the niche cells and integrin-mediated cell-to-ECM adhesion between the stem cells and the basement membrane are two general types of cell

adhesion utilized in the stem cell niches. In addition to the role of adhesion molecules in anchoring stem cells, they also participate in regulating stem cell division by anchoring and orientating mitotic spindles and regulating signaling cascades (Marthiens et al. 2010; Xi 2009).

3.6 Classification of Stem Cell Niches: Stromal Versus Epidermal

Based on the comparison of physical structures among these well-characterized stem cell niches in simple organisms, the niche can be categorized into two general types, stromal niche and epidermal niche (Morrison and Spradling 2008), which may also be applicable to the stem cell niches in mammals.

The stromal niche is best exemplified by the GSC niches in *Drosophila*. The stromal niche is constituted of fixed stromal cells. For example, cap cells or hub cells constitute the female and male GSC niches, respectively. In the stromal niche, the stem cells are usually anchored to the niche cells by forming cadherin-mediated adherens junctions. The junctional structure at the stem cell-niche interface may be utilized for spindle pole anchorage for asymmetric stem cell division. In the stromal niche, short range self-renewal signals from the niche cells are critical for stem cell self-renewal, such that stem cells that are out of the niche could not receive self-renewal signals and will commit differentiation. On the other hand, stem cells could also send signals back to the niche cells to maintain their fate and function (Fig. 3.3a).

In the epidermal niche, exemplified by the FSC niche in the *Drosophila* ovary and the ISC niche in the *Drosophila* midgut, stem cells do not directly contact any fixed stromal cells but are constantly associated with the basement membrane composed of ECM. In addition, both stem cells and their differentiating daughter cells are exposed to seemingly similar surrounding environments without apparently distinctive compartalization. Stem cell anchorage and self-renewal mechanisms are different from that utilized in the stromal niche, and may be diverse from one system to another (Fig. 3.3b). In the FSC niche, stem cells are anchored in a fixed location by integrin-mediated cell adhesion between the stem cell and the ECM. Stem cells receive multiple signals produced from a relative distant source at the anterior tip for their self-renewal. There is no evidence for a specific composition of ECM at the stem cell location and the location of the FSC is probably controlled by both the levels of self-renewal signaling activity and communications between the stem cells and nearby non-stem cells and ECM. In the single-layered *Drosophila* midgut epithelium, ISCs are lining along the basement membrane that separates the epithelial layer with the muscular niche. The non-stem epithelial cells including enterocytes and enteroendocrine cells are also in direct contact with the basement membrane, and Wingless and Unpaired self-renewal signals are expressed in the muscle cells along the length of the midgut. Thus, it seems that in addition to ISCs, non-stem epithelial cells are also exposed to the niche microenvironment. It is therefore

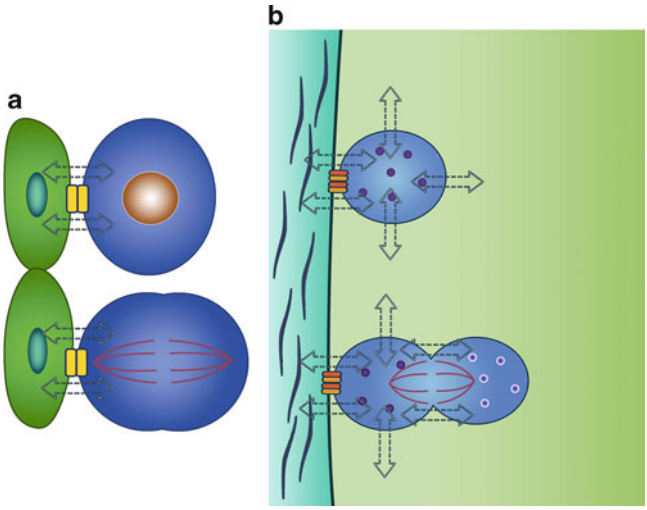


Fig. 3.3 Classification of stem cell niches based on cellular and structural composition. (**a**) A stromal niche. In the stromal niche, stem cells are anchored in the niche cells by forming cadherin-mediated cell-to-cell adhesion between the stem cells and the niche cells. Signaling between the niche cells and the stem cells is critical for stem cell maintenance and self-renewal. (**b**) An epidermal niche. In the epidermal niche, stem cells are anchored in the niche by forming integrin-mediated cell-to-ECM adhesion between the stem cells and the basement membrane. Signaling interactions between the stem cells and the niche environment, including the ECM, the neighboring cells and the immediate daughters may cooperatively regulate stem cell fate or symmetric or asymmetric segregation of cell fate determinants

possible that stem cell self-renewal could be controlled by additional mechanisms in addition to the instructive signals from the muscular niche. Delta expressing ISC could direct daughter cell fate by activation of Notch in the differentiating daughter cells, and Delta-Notch mediated lateral inhibition may further reinforce each other's cell fate. Thus, stem cell self-renewal in the epidermal niche is possibly controlled by both the instructive communications between the stem cells and the niche, and the instructive communications between the stem cells and neighboring differentiated cells, including the differentiating daughter cells (Fig. 3.3b).

3.7 Stem Cell Self-renewal in the Niche: Division Asymmetry Versus Population Asymmetry

As the ultimate defense for tissue homeostasis, stem cells have to accomplish two tasks throughout adult life: one is to generate more stem cells (self-renewal), the other is to produce committed cells (differentiation). And these two tasks must be tightly coordinated. Accumulating data from studies in invertebrates together with vertebrates point out two plausible strategies used by stem cells to interpret how the balance between

self-renewal and differentiation is achieved. Stem cells can adopt either division asymmetry or population asymmetry strategy to maintain tissue homeostasis (Morrison and Kimble 2006; Simons and Clevers 2011; Watt and Hogan 2000).

Division asymmetry refers to that each individual stem cell divides to produce two daughters with distinct fates: one remains as a new stem cell and the other commits differentiation. Asymmetric division can be achieved either through asymmetric segregation of cell fate determinants, such as for Drosophila neuroblasts (Knoblich 2008), or through cues from the niche. The well-characterized *Drosophila* GSCs in the ovary and testis use the latter strategy. In this scenario, the highly asymmetric niche architecture directs and facilitates the outcome of stem cell division: the daughter cell remained in the niche will self-renewal, while the daughter cell positioned away from the niche will differentiate.

In population asymmetry, each stem cell gives rise to two daughter cells upon division, the fate of which is unpredictable and depends on the extrinsic input. Some stem cells may be lost through differentiation and some stem cells can expand to replace the lost stem cells. And the replacement rate is comparable to the loss rate. Therefore, the net effect of population asymmetry is the same as division asymmetry. The total number of stem cells remains constant at the level of stem cell population. Stem cells in many mammalian tissues adopt this strategy to achieve homeostasis. For instance, the $Lgr5^{hi}$ ISCs in mouse intestine divide symmetrically to generate two daughter cells, which subsequently undergo "neutral competition" for contact with Paneth cells with the neighboring stem cells. And the loser cells in the competition are squeezed out of the niche to initiate the differentiation program (Lopez-Garcia et al. 2010; Snippert et al. 2010). Besides, the GSCs in mammalian testis and epidermal stem cells in mouse interfollicle epithelium might fall into this category as well.

3.8 Stem Cell Behavior Within the Niche

Studies on the *Drosophila* GSC niche have also revealed several interesting stem cell behaviors that may be important for stem cell long-term maintenance and function, and those phenomena have enriched our understanding of the stem cell niche concept. Here are some examples.

1. Stem cell replacement. It is evident that adult stem cells have limited half-life. They turn over regularly, but the stem cell number within each niche could remain relatively constant. This is probably due to a phenomenon named stem cell replacement. One example is the GSC in the *Drosophila* ovary. When one GSC is depleted from the niche, the other GSCs could undergo symmetric division to supplement the lost GSC (Xie and Spradling 2000). This indicates that the niche has the capability to sustain a stable number of GSCs by controlling symmetric and asymmetric division of GSCs.
2. Stem cell dedifferentiation in the niche. This represents another potentially important mechanism for maintaining constant stem cell number in the niche.

When GSCs in the *Drosophila* ovary and testis are forced to differentiate, the early differentiating germ cells could be dedifferentiated into functional GSCs and reoccupy the niche, if they again receive the niche signaling. This reveals the plasticity of progenitor cells and a dominant role of niche in determining stem cell fate (Brawley and Matunis 2004; Kai and Spradling 2004).

3. Stem cell competition. The regular turn-over of stem cells and replacement by the neighboring stem cells may also indicate that these stem cells within the same niche may constantly compete with each other for niche occupation. Studies of GSCs with different genetic background in the same niche have shown that cell adhesion molecules are involved in stem cell competition (Jin et al. 2008). Stem cell competition may be important for the quality control of stem cells, and for coordinating the functions of different types of stem cells that share a single niche (Rhiner et al. 2009). It is also possible that cancer stem cells could potentially make more devastating damages by utilizing this mechanism to hijack the niche and eliminate the normal stem cells.

3.9 Future Perspective

The study of the stem cells and their niches has provided important implications on the relationships between dysregulation of the stem cell niche and human diseases and aging, and may provide useful strategies for clinical applications. Increasing evidence suggests that many cancers are stem cell diseases, in which a rare population of cancer stem cells is responsible for the initiation and recurrence of cancers (Clarke and Fuller 2006). Understanding stem cell self-renewal mechanisms could help to provide novel therapeutic strategy to treat cancers. For example, the CD44 adhesion receptor, which is known to mediate Osteopontin signaling from the niche, could be a therapeutic target of acute myeloid leukemia (AML) cancer stem cells, as administration of CD44 antibody efficiently eliminates leukemia stem cells in the mouse model of human AML (Jin et al. 2006). In addition, abnormalities in the niche, rather than stem cells themselves, may also lead to the development of cancers. For example, increasing evidence suggests that leukemia could be contributed by both cell autonomous abnormalities and dysfunction of the microenvironment in the bone marrow (Lane et al. 2009). Microenvironmental deletion of retinoic acid gamma receptor (RARγ) or retinoblastoma leads to a phenotype reminiscent of myeloproliferative disease in mouse, which raises the possibility that some leukemia may result from disorder of the microenvironment (Walkley et al. 2007). Therefore, targeting abnormal niche function could be another therapeutic strategy to treat cancers.

Understanding of the stem cell and niche regulation may also lead to improved methods for stem cell manipulation in vivo and in vitro to facilitate replacement therapies in the future. For example, osteoblastic cells, the niche cells for HSCs, can be manipulated by PTH in mouse models of clinical use of HSCs. PTH administration

can increase stem cell harvest, protect HSC from chemotherapy and promote HSC function in transplant recipients (Adams et al. 2007).

The ability of adult stem cells to regenerate tissue declines with age and this phenomenon, regarded as stem cell aging, is contributed by the changes in the niche microenvironment, systemic environment and intrinsically within the stem cells, although the contribution of each factor could vary greatly in different tissues and organisms. For example, in the *Drosophila* testis and ovary, the GSC activity declines greatly with age, largely due to the functional decay of niche signaling (Boyle et al. 2007; Pan et al. 2007; Zhao et al. 2008). In mouse satellite stem cell niche, systemic change-induced Wnt signaling activation has been linked to the decline of regeneration potential in aged satellite stem cells (Brack et al. 2007; Carlson et al. 2008). Therefore, modulating stem cell niche function could also be a useful strategy to delay the development of aging and promote tissue regeneration and damage repair.

Aside from these promising clinical prospective, there are still a lot of mysteries about the stem cells and their associated niches. The identification and characterization of these less understood mammalian stem cell niches would be an urgent task. How the extrinsic signals integrate with intrinsic circuitries to maintain the stemness and how stem cell self-renewal and differentiation are precisely balanced only begins to be understood. Again, studies on simpler genetic model systems would certainly continue to pioneer our understanding of stem cells and their niches.

References

Adams GB, Martin RP, Alley IR, Chabner KT, Cohen KS, Calvi LM, Kronenberg HM, Scadden DT (2007) Therapeutic targeting of a stem cell niche. Nat Biotechnol 25:238–243

Andreu P, Colnot S, Godard C, Gad S, Chafey P, Niwa-Kawakita M, Laurent-Puig P, Kahn A, Robine S, Perret C et al (2005) Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. Development 132:1443–1451

Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118:149–161

Asahina M, Valenta T, Silhankova M, Korinek V, Jindra M (2006) Crosstalk between a nuclear receptor and beta-catenin signaling decides cell fates in the C. elegans somatic gonad. Dev Cell 11:203–211

Austin J, Kimble J (1987) glp-1 is required in the germ line for regulation of the decision between mitosis and meiosis in C. elegans. Cell 51:589–599

Barker N (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449:1003–1007

Beebe K, Lee WC, Micchelli CA (2009) JAK/STAT signaling coordinates stem cell proliferation and multilineage differentiation in the Drosophila intestinal stem cell lineage. Dev Biol 338:28–37

Berry LW, Westlund B, Schedl T (1997) Germ-line tumor formation caused by activation of glp-1, a Caenorhabditis elegans member of the Notch family of receptors. Development 124:925–936

Biteau B, Jasper H (2011) EGF signaling regulates the proliferation of intestinal stem cells in Drosophila. Development 138:1045–1055

Blanpain C, Fuchs E (2006) Epidermal stem cells of the skin. Annu Rev Cell Dev Biol 22:339–373

Blanpain C, Fuchs E (2009) Epidermal homeostasis: a balancing act of stem cells in the skin. Nat Rev Mol Cell Biol 10:207–217

Blanpain C, Lowry WE, Geoghegan A, Polak L, Fuchs E (2004) Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 118:635–648

Blessing M, Nanney LB, King LE, Jones CM, Hogan BL (1993) Transgenic mice as a model to study the role of TGF-beta-related molecules in hair follicles. Genes Dev 7:204–215

Botchkarev VA, Botchkareva NV, Roth W, Nakamura M, Chen LH, Herzog W, Lindner G, McMahon JA, Peters C, Lauster R et al (1999) Noggin is a mesenchymally derived stimulator of hair-follicle induction. Nat Cell Biol 1:158–164

Boyle M, Wong C, Rocha M, Jones DL (2007) Decline in self-renewal factors contributes to aging of the stem cell niche in the Drosophila testis. Cell Stem Cell 1:470–478

Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, Rando TA (2007) Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science 317:807–810

Brack AS, Conboy IM, Conboy MJ, Shen J, Rando TA (2008) A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. Cell Stem Cell 2:50–59

Brawley C, Matunis E (2004) Regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo. Science 304:1331–1334

Breault DT (2008) Generation of mTert-GFP mice as a model to identify and study tissue progenitor cells. Proc Natl Acad Sci USA 105:10420–10425

Brownell I, Guevara E, Bai CB, Loomis CA, Joyner AL (2011) Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. Cell Stem Cell 8:552–565

Buchon N, Broderick NA, Chakrabarti S, Lemaitre B (2009) Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila. Genes Dev 23:2333–2344

Buchon N, Broderick NA, Kuraishi T, Lemaitre B (2010) Drosophila EGFR pathway coordinates stem cell proliferation and gut remodeling following infection. BMC Biol 8:152

Byrd DT, Kimble J (2009) Scratching the niche that controls Caenorhabditis elegans germline stem cells. Semin Cell Dev Biol 20:1107–1113

Calvi LM (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425:841–846

Carlson ME, Hsu M, Conboy IM (2008) Imbalance between pSmad3 and Notch induces CDK inhibitors in old muscle stem cells. Nature 454:528–532

Chen D, McKearin D (2003) Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. Curr Biol 13:1786–1791

Chen S, Wang S, Xie T (2011) Restricting self-renewal signals within the stem cell niche: multiple levels of control. Curr Opin Genet Dev 21(6):684–689

Chesney MA, Lam N, Morgan DE, Phillips BT, Kimble J (2009) C. elegans HLH-2/E/Daughterless controls key regulatory cells during gonadogenesis. Dev Biol 331:14–25

Clarke MF, Fuller M (2006) Stem cells and cancer: two faces of eve. Cell 124:1111–1115

Conboy IM, Rando TA (2002) The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell 3:397–409

Conboy IM, Conboy MJ, Smythe GM, Rando TA (2003) Notch-mediated restoration of regenerative potential to aged muscle. Science 302:1575–1577

Cox DN, Chao A, Baker J, Chang L, Qiao D, Lin H (1998) A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev 12:3715–3727

Crittenden SL, Troemel ER, Evans TC, Kimble J (1994) GLP-1 is localized to the mitotic region of the C. elegans germ line. Development 120:2901–2911

Crittenden SL, Bernstein DS, Bachorik JL, Thompson BE, Gallegos M, Petcherski AG, Moulder G, Barstead R, Wickens M, Kimble J (2002) A conserved RNA-binding protein controls germline stem cells in Caenorhabditis elegans. Nature 417:660–663

Crittenden SL, Leonhard KA, Byrd DT, Kimble J (2006) Cellular analyses of the mitotic region in the Caenorhabditis elegans adult germ line. Mol Biol Cell 17:3051–3061

DasGupta R, Fuchs E (1999) Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. Development 126:4557–4568

Decotto E, Spradling AC (2005) The Drosophila ovarian and testis stem cell niches: similar somatic stem cells and signals. Dev Cell 9:501–510

Dinardo S, Okegbe T, Wingert L, Freilich S, Terry N (2011) Lines and bowl affect the specification of cyst stem cells and niche cells in the Drosophila testis. Development 138:1687–1696

Eckmann CR, Crittenden SL, Suh N, Kimble J (2004) GLD-3 and control of the mitosis/meiosis decision in the germline of Caenorhabditis elegans. Genetics 168:147–160

Eliazer S, Shalaby NA, Buszczak M (2011) Loss of lysine-specific demethylase 1 nonautonomously causes stem cell tumors in the Drosophila ovary. Proc Natl Acad Sci U S A 108:7064–7069

Festa E, Fretz J, Berry R, Schmidt B, Rodeheffer M, Horowitz M, Horsley V (2011) Adipocyte lineage cells contribute to the skin stem cell niche to drive hair cycling. Cell 146:761–771

Fleming HE, Janzen V, Lo Celso C, Guo J, Leahy KM, Kronenberg HM, Scadden DT (2008) Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. Cell Stem Cell 2:274–283

Forbes AJ, Lin H, Ingham PW, Spradling AC (1996) hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in Drosophila. Development 122:1125–1135

Fuchs E (2009) The tortoise and the hair: slow-cycling cells in the stem cell race. Cell 137:811–819

Gat U, DasGupta R, Degenstein L, Fuchs E (1998) De novo hair follicle morphogenesis and hair tumors in mice expressing a truncated [beta]-catenin in skin. Cell 95:605–614

Girgenrath M, Weng S, Kostek CA, Browning B, Wang M, Brown SA, Winkles JA, Michaelson JS, Allaire N, Schneider P et al (2006) TWEAK, via its receptor Fn14, is a novel regulator of mesenchymal progenitor cells and skeletal muscle regeneration. EMBO J 25:5826–5839

Guo Z, Wang Z (2009) The glypican Dally is required in the niche for the maintenance of germline stem cells and short-range BMP signaling in the Drosophila ovary. Development 136:3627–3635

Haramis AP, Begthel H, van den Born M, van Es J, Jonkheer S, Offerhaus GJ, Clevers H (2004) De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. Science 303:1684–1686

Hayashi Y, Kobayashi S, Nakato H (2009) Drosophila glypicans regulate the germline stem cell niche. J Cell Biol 187:473–480

He XC, Zhang J, Tong WG, Tawfik O, Ross J, Scoville DH, Tian Q, Zeng X, He X, Wiedemann LM et al (2004) BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. Nat Genet 36:1117–1121

Henderson ST, Gao D, Lambie EJ, Kimble J (1994) lag-2 may encode a signaling ligand for the GLP-1 and LIN-12 receptors of C. elegans. Development 120:2913–2924

Hooper AT, Butler JM, Nolan DJ, Kranz A, Iida K, Kobayashi M, Kopp HG, Shido K, Petit I, Yanger K et al (2009) Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. Cell Stem Cell 4:263–274

Huelsken J, Vogel R, Erdmann B, Cotsarelis G, Birchmeier W (2001) beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell 105:533–545

Jiang H, Patel PH, Kohlmaier A, Grenley MO, McEwen DG, Edgar BA (2009) Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell 137:1343–1355

Jiang H, Grenley MO, Bravo MJ, Blumhagen RZ, Edgar BA (2010) EGFR/Ras/MAPK signaling mediates adult midgut epithelial homeostasis and regeneration in Drosophila. Cell Stem Cell 8:84–95

Jin L, Hope KJ, Zhai Q, Smadja-Joffe F, Dick JE (2006) Targeting of CD44 eradicates human acute myeloid leukemic stem cells. Nat Med 12:1167–1174

Jin Z, Kirilly D, Weng C, Kawase E, Song X, Smith S, Schwartz J, Xie T (2008) Differentiation-defective stem cells outcompete normal stem cells for niche occupancy in the Drosophila ovary. Cell Stem Cell 2:39–49

Kai T, Spradling A (2004) Differentiating germ cells can revert into functional stem cells in Drosophila melanogaster ovaries. Nature 428:564–569

Karp X, Greenwald I (2004) Multiple roles for the E/Daughterless ortholog HLH-2 during C. elegans gonadogenesis. Dev Biol 272:460–469

Kawase E, Wong MD, Ding BC, Xie T (2004) Gbb/Bmp signaling is essential for maintaining germline stem cells and for repressing bam transcription in the Drosophila testis. Development 131:1365–1375

Kidd AR 3rd, Miskowski JA, Siegfried KR, Sawa H, Kimble J (2005) A beta-catenin identified by functional rather than sequence criteria and its role in Wnt/MAPK signaling. Cell 121:761–772

Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ (2005) SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121:1109–1121

Kiel MJ, Acar M, Radice GL, Morrison SJ (2009) Hematopoietic stem cells do not depend on N-cadherin to regulate their maintenance. Cell Stem Cell 4:170–179

Kiger AA, Jones DL, Schulz C et al (2001) Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. Science 294:2542–2545

Kimble J, Crittenden SL (2007) Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in Caenorhabditis elegans. Annu Rev Cell Dev Biol 23:405–433

Kimble JE, White JG (1981) On the control of germ cell development in Caenorhabditis elegans. Dev Biol 81:208–219

King FJ, Lin H (1999) Somatic signaling mediated by fs(1)Yb is essential for germline stem cell maintenance during Drosophila oogenesis. Development 126:1833–1844

Kirilly D, Spana EP, Perrimon N, Padgett RW, Xie T (2005) BMP signaling is required for controlling somatic stem cell self-renewal in the Drosophila ovary. Dev Cell 9:651–662

Kitadate Y, Kobayashi S (2010) Notch and Egfr signaling act antagonistically to regulate germ-line stem cell niche formation in Drosophila male embryonic gonads. Proc Natl Acad Sci U S A 107:14241–14246

Knoblich JA (2008) Mechanisms of asymmetric stem cell division. Cell 132:583–597

Kobielak K, Stokes N, de la Cruz J et al (2007) Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. Proc Natl Acad Sci USA 104:10063–10068

Korinek V, Barker N, Moerer P, van Donselaar E, Huls G, Peters PJ, Clevers H (1998) Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet 19:379–383

Kuang S, Kuroda K, Le Grand F, Rudnicki MA (2007) Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell 129:999–1010

Kuang S, Gillespie MA, Rudnicki MA (2008) Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell 2:22–31

LaBarge MA, Blau HM (2002) Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. Cell 111:589–601

Lam N, Chesney MA, Kimble J (2006) Wnt signaling and CEH-22/tinman/Nkx2.5 specify a stem cell niche in C. elegans. Curr Biol 16:287–295

Lambie EJ, Kimble J (1991) Two homologous regulatory genes, lin-12 and glp-1, have overlapping functions. Development 112:231–240

Lane SW, Scadden DT, Gilliland DG (2009) The leukemic stem cell niche: current concepts and therapeutic opportunities. Blood 114:1150–1157

Leatherman JL, Dinardo S (2008) Zfh-1 controls somatic stem cell self-renewal in the Drosophila testis and nonautonomously influences germline stem cell self-renewal. Cell Stem Cell 3:44–54
Leatherman JL, Dinardo S (2010) Germline self-renewal requires cyst stem cells and stat regulates niche adhesion in Drosophila testes. Nat Cell Biol 12:806–811
Lee WC, Beebe K, Sudmeier L, Micchelli CA (2009) Adenomatous polyposis coli regulates Drosophila intestinal stem cell proliferation. Development 136:2255–2264
Li X, Han Y, Xi R (2010) Polycomb group genes Psc and Su(z)2 restrict follicle stem cell self-renewal and extrusion by controlling canonical and noncanonical Wnt signaling. Genes Dev 24:933–946
Lin G, Xu N, Xi R (2008) Paracrine wingless signalling controls self-renewal of Drosophila intestinal stem cells. Nature 455:1119–1123
Lin G, Xu N, Xi R (2009) Paracrine unpaired signaling through the JAK/STAT pathway controls self-renewal and lineage differentiation of drosophila intestinal stem cells. J Mol Cell Biol 2:37–49
Liu W, Singh SR, Hou SX (2010) JAK-STAT is restrained by Notch to control cell proliferation of the Drosophila intestinal stem cells. J Cell Biochem 109:992–999
Lopez-Garcia C, Klein AM, Simons BD, Winton DJ (2010) Intestinal stem cell replacement follows a pattern of neutral drift. Science 330:822–825
Lopez-Onieva L, Fernandez-Minan A, Gonzalez-Reyes A (2008) Jak/Stat signalling in niche support cells regulates dpp transcription to control germline stem cell maintenance in the Drosophila ovary. Development 135:533–540
Losick VP, Morris LX, Fox DT, Spradling A (2011) Drosophila stem cell niches: a decade of discovery suggests a unified view of stem cell regulation. Dev Cell 21:159–171
Lowry WE, Blanpain C, Nowak JA, Guasch G, Lewis L, Fuchs E (2005) Defining the impact of beta-catenin/Tcf transactivation on epithelial stem cells. Genes Dev 19:1596–1611
Madison BB, Braunstein K, Kuizon E, Portman K, Qiao XT, Gumucio DL (2005) Epithelial hedgehog signals pattern the intestinal crypt-villus axis. Development 132:279–289
Marthiens V, Kazanis I, Moss L, Long K, ffrench-Constant C (2010) Adhesion molecules in the stem cell niche ? more than just staying in shape? J Cell Sci 123:1613–1622
Mendez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466:829–834
Micchelli CA, Perrimon N (2006) Evidence that stem cells reside in the adult Drosophila midgut epithelium. Nature 439:475–479
Mitchell KJ, Pannerec A, Cadot B, Parlakian A, Besson V, Gomes ER, Marazzi G, Sassoon DA (2010) Identification and characterization of a non-satellite cell muscle resident progenitor during postnatal development. Nat Cell Biol 12:257–266
Miyagoe Y, Hanaoka K, Nonaka I, Hayasaka M, Nabeshima Y, Arahata K, Takeda S (1997) Laminin alpha2 chain-null mutant mice by targeted disruption of the Lama2 gene: a new model of merosin (laminin 2)-deficient congenital muscular dystrophy. FEBS Lett 415:33–39
Montgomery RK (2011) Mouse telomerase reverse transcriptase (mTert) expression marks slowly cycling intestinal stem cells. Proc Natl Acad Sci USA 108:179–184
Moriyama M, Durham AD, Moriyama H, Hasegawa K, Nishikawa S, Radtke F, Osawa M (2008) Multiple roles of Notch signaling in the regulation of epidermal development. Dev Cell 14:594–604
Morris LX, Spradling AC (2011) Long-term live imaging provides new insight into stem cell regulation and germline-soma coordination in the Drosophila ovary. Development 138:2207–2215
Morrison SJ, Kimble J (2006) Asymmetric and symmetric stem-cell divisions in development and cancer. Nature 441:1068–1074
Morrison SJ, Spradling AC (2008) Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell 132:598–611

Nadarajan S, Govindan JA, McGovern M, Hubbard EJ, Greenstein D (2009) MSP and GLP-1/Notch signaling coordinately regulate actomyosin-dependent cytoplasmic streaming and oocyte growth in C. elegans. Development 136:2223–2234

Nie Y, Han YC, Zou YR (2008) CXCR4 is required for the quiescence of primitive hematopoietic cells. J Exp Med 205:777–783

Nilsson SK, Johnston HM, Whitty GA, Williams B, Webb RJ, Denhardt DT, Bertoncello I, Bendall LJ, Simmons PJ, Haylock DN (2005) Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood 106:1232–1239

Nystul T, Spradling A (2007) An epithelial niche in the Drosophila ovary undergoes long-range stem cell replacement. Cell Stem Cell 1:277–285

O'Brien LE, Soliman SS, Li X, Bilder D (2011) Altered modes of stem cell division drive adaptive intestinal growth. Cell 147:603–614

Ohlstein B, Spradling A (2006) The adult Drosophila posterior midgut is maintained by pluripotent stem cells. Nature 439:470–474

Ohlstein B, Spradling A (2007) Multipotent Drosophila intestinal stem cells specify daughter cell fates by differential notch signaling. Science 315:988–992

Omatsu Y, Sugiyama T, Kohara H, Kondoh G, Fujii N, Kohno K, Nagasawa T (2010) The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. Immunity 33:387–399

O'Reilly AM, Lee HH, Simon MA (2008) Integrins control the positioning and proliferation of follicle stem cells in the Drosophila ovary. J Cell Biol 182:801–815

Pan L, Chen S, Weng C, Call G, Zhu D, Tang H, Zhang N, Xie T (2007) Stem cell aging is controlled both intrinsically and extrinsically in the Drosophila ovary. Cell Stem Cell 1:458–469

Reddy S, Andl T, Bagasra A, Lu MM, Epstein DJ, Morrisey EE, Millar SE (2001) Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis. Mech Dev 107:69–82

Rhiner C, Diaz B, Portela M, Poyatos JF, Fernandez-Ruiz I, Lopez-Gay JM, Gerlitz O, Moreno E (2009) Persistent competition among stem cells and their daughters in the Drosophila ovary germline niche. Development 136:995–1006

Sangiorgi E, Capecchi MR (2008) Bmi1 is expressed in vivo in intestinal stem cells. Nat Genet 40:915–920

Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Batlle E, Simon-Assmann P, Clevers H, Nathke IS et al (2004) Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev 18:1385–1390

Sato T (2009) Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459:262–265

Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, Barker N, Shroyer NF, van de Wetering M, Clevers H (2011) Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 469:415–418

Schepers AG, Vries R, van den Born M, van de Wetering M, Clevers H (2011) Lgr5 intestinal stem cells have high telomerase activity and randomly segregate their chromosomes. EMBO J 30:1104–1109

Schofield R (1978) The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 4:7–25

Sick S, Reinker S, Timmer J, Schlake T (2006) WNT and DKK determine hair follicle spacing through a reaction–diffusion mechanism. Science 314:1447–1450

Siegfried KR, Kimble J (2002) POP-1 controls axis formation during early gonadogenesis in C. elegans. Development 129:443–453

Siegfried KR, Kidd AR 3rd, Chesney MA, Kimble J (2004) The sys-1 and sys-3 genes cooperate with Wnt signaling to establish the proximal-distal axis of the Caenorhabditis elegans gonad. Genetics 166:171–186

Simons BD, Clevers H (2011) Strategies for homeostatic stem cell self-renewal in adult tissues. Cell 145:851–862

Sipkins DA, Wei X, Wu JW, Runnels JM, Cote D, Means TK, Luster AD, Scadden DT, Lin CP (2005) In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature 435:969–973

Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, Barker N, Klein AM, van Rheenen J, Simons BD et al (2010) Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. Cell 143:134–144

Song X, Xie T (2002) DE-cadherin-mediated cell adhesion is essential for maintaining somatic stem cells in the Drosophila ovary. Proc Natl Acad Sci U S A 99:14813–14818

Song X, Xie T (2003) Wingless signaling regulates the maintenance of ovarian somatic stem cells in Drosophila. Development 130:3259–3268

Song X, Zhu CH, Doan C, Xie T (2002) Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. Science 296:1855–1857

Song X, Wong MD, Kawase E, Xi R, Ding BC, McCarthy JJ, Xie T (2004) Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. Development 131:1353–1364

Song X, Call GB, Kirilly D, Xie T (2007) Notch signaling controls germline stem cell niche formation in the Drosophila ovary. Development 134:1071–1080

Stier S, Ko Y, Forkert R, Lutz C, Neuhaus T, Grunewald E, Cheng T, Dombkowski D, Calvi LM, Rittling SR et al (2005) Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J Exp Med 201:1781–1791

Sugiyama T, Kohara H, Noda M, Nagasawa T (2006) Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity 25:977–988

Suh N, Crittenden SL, Goldstrohm A, Hook B, Thompson B, Wickens M, Kimble J (2009) FBF and its dual control of gld-1 expression in the Caenorhabditis elegans germline. Genetics 181:1249–1260

Taichman RS, Reilly MJ, Emerson SG (1996) Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures. Blood 87:518–524

Tatsumi R, Anderson JE, Nevoret CJ, Halevy O, Allen RE (1998) HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. Dev Biol 194:114–128

Tian H, Biehs B, Warming S, Leong KG, Rangell L, Klein OD, de Sauvage FJ (2011) A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. Nature 478(7368):255–259

Tulina N, Matunis E (2001) Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. Science 294:2546–2549

van den Brink GR, Bleuming SA, Hardwick JC, Schepman BL, Offerhaus GJ, Keller JJ, Nielsen C, Gaffield W, van Deventer SJ, Roberts DJ et al (2004) Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. Nat Genet 36:277–282

van der Flier LG, Clevers H (2009) Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol 71:241–260

Visnjic D, Kalajzic Z, Rowe DW, Katavic V, Lorenzo J, Aguila HL (2004) Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood 103:3258–3264

Voog J, Jones DL (2010) Stem cells and the niche: a dynamic duo. Cell Stem Cell 6:103–115

Voog J, D'Alterio C, Jones DL (2008) Multipotent somatic stem cells contribute to the stem cell niche in the Drosophila testis. Nature 454:1132–1136

Walkley CR, Olsen GH, Dworkin S, Fabb SA, Swann J, McArthur GA, Westmoreland SV, Chambon P, Scadden DT, Purton LE (2007) A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. Cell 129:1097–1110

Wang X, Harris RE, Bayston LJ, Ashe HL (2008) Type IV collagens regulate BMP signalling in Drosophila. Nature 455:72–77

Ward EJ, Shcherbata HR, Reynolds SH, Fischer KA, Hatfield SD, Ruohola-Baker H (2006) Stem cells signal to the niche through the Notch pathway in the Drosophila ovary. Curr Biol 16:2352–2358

Watt FM (1998) Epidermal stem cells: markers, patterning and the control of stem cell fate. Philos Trans R Soc Lond B Biol Sci 353:831–837
Watt FM, Hogan BL (2000) Out of Eden: stem cells and their niches. Science 287:1427–1430
Xi R (2009) Anchoring stem cells in the niche by cell adhesion molecules. Cell Adhes Migr 3:396–401
Xi R, Xie T (2005) Stem cell self-renewal controlled by chromatin remodeling factors. Science 310:1487–1489
Xia L, Jia S, Huang S, Wang H, Zhu Y, Mu Y, Kan L, Zheng W, Wu D, Li X et al (2010) The Fused/Smurf complex controls the fate of Drosophila germline stem cells by generating a gradient BMP response. Cell 143:978–990
Xie T, Spradling AC (1998) decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. Cell 94:251–260
Xie T, Spradling AC (2000) A niche maintaining germ line stem cells in the Drosophila ovary. Science 290:328–330
Xu N, Wang SQ, Tan D, Gao Y, Lin G, Xi R (2011) EGFR, Wingless and JAK/STAT signaling cooperatively maintain Drosophila intestinal stem cells. Dev Biol 354:31–43
Yamashita YM, Jones DL, Fuller MT (2003) Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. Science 301:1547–1550
Yamashita YM, Mahowald AP, Perlin JR, Fuller MT (2007) Asymmetric inheritance of mother versus daughter centrosome in stem cell division. Science 315:518–521
Yi R, Poy MN, Stoffel M, Fuchs E (2008) A skin microRNA promotes differentiation by repressing 'stemness'. Nature 452:225–229
Ying QL, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A (2008) The ground state of embryonic stem cell self-renewal. Nature 453:519–523
Yoshihara H, Arai F, Hosokawa K, Hagiwara T, Takubo K, Nakamura Y, Gomei Y, Iwasaki H, Matsuoka S, Miyamoto K et al (2007) Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell Stem Cell 1:685–697
Zhang J (2003) Identification of the haematopoietic stem cell niche and control of the niche size. Nature 425:836–841
Zhang Y, Kalderon D (2001) Hedgehog acts as a somatic stem cell factor in the Drosophila ovary. Nature 410:599–604
Zhang J, Li L (2008) Stem cell niche: microenvironment and beyond. J Biol Chem 283:9499–9503
Zhao R, Xuan Y, Li X, Xi R (2008) Age-related changes of germline stem cell activity, niche signaling activity and egg production in Drosophila. Aging Cell 7:344–354
Zhu L (2009) Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. Nature 457:603–607

Chapter 4
Stem Cells and Asymmetric Cell Division

Frank Hirth

Abstract Asymmetric stem cell division is a fundamental process used to generate cellular diversity and to provide a source of new cells in developing and adult organisms. Asymmetric stem cell division leads to another stem cell via self-renewal, and a second cell type which can be either a differentiating progenitor or a postmitotic cell. Experimental studies in model organisms including the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster* and the laboratory mouse, *Mus musculus*, have identified interrelated mechanisms that regulate asymmetric stem cell division from polarity formation and mitotic spindle orientation to asymmetric segregation of cell fate determinants and growth control. These mechanisms are mediated by evolutionary conserved molecules including Aurora-A, aPKC, Mud/NuMa, Lgl, Numb and Brat/TRIM-NHL, which in turn regulate a binary switch between stem cell self-renewal and differentiation. The mechanistic insights into asymmetric cell division have enhanced our understanding of stem cell biology and are of major therapeutic interest for regenerative medicine as asymmetrically dividing stem cells provide a powerful source for targeted cell replacement and tissue regeneration.

Abbreviations

Ago1	Argonaute protein 1
AurA	Aurora-A
Baz	Bazooka
Brat	Brain tumor

F. Hirth, B.Sc., M.Sc., Ph.D., PD (✉)
Department of Neuroscience, MRC Centre for Neurodegeneration Research,
Institute of Psychiatry, King's College London, PO Box 37, De Crespigny Park,
Denmark Hill, London, SE5 8AF, UK
e-mail: Frank.Hirth@kcl.ac.uk

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_4,

Cdc2	Cell division cycle 2
Cdc42	Cell division cycle 42
Cdc25	Cell division cycle 25
Cdk	cyclin dependent kinase
Cnn	centrosomin
CNS	Central Nervous System
c-Myc	cellular myelocytomatosis oncogene
DaPKC	Drosophila atypical protein kinase C
Dctn1	dynactin
Dlg	Discs large
DmPar6	Drosophila melanogaster Partitioning defective 6
ESC	embryonic stem cell
ECT-2	epithelial cell transforming gene 2
Galphai	G-protein alpha, subunit i
GMC	Ganglion Mother Cell
GoLoco	G-protein 0, Locomotion defects domain
GDPase	guanosine diphosphatase
GTPase	guanosine triphosphatase
Insc	Inscuteable
Khc-73	Kinesin heavy chain 73
Lgl	Lethal (2) giant larvae
Mira	Miranda
Mud	Mushroom body defect
NB	Neuroblast
NHL	NCL-1, HT2A, and LIN-41 domain
NuMa	Nuclear Mitotic apparatus
PAR	partitioning defective
Par-3	partitioning defective 3
Par-6	partitioning defective 6
PDZ	Post synaptic density 95, Discs large, and Zonula occludens-1 domain
Pins	Partner of Inscuteable
Pon	Partner of Numb
Pros	Prospero
RNA	Ribonucleic Acid
Sqh	Spaghetti squash
TRIM 3	tripartite motif protein 3
TRIM 32	tripartite motif protein 32
VNC	Ventral Nerve Cord

4.1 Introduction

Stem cells are characterised by their potential to self-renew and to differentiate into every cell type of the organism. Stem cells are found in developing and adult tissue, starting with the totipotent zygote which subsequently leads to

pluripotent stem cells of the early embryo. Later during germ layer formation and organogenesis, stem cells become increasingly restricted in their lineage potential and give rise to progeny that contribute to mature tissue (Eckfeldt et al. 2005; Slack 2008; Murry and Keller 2008; Metallo et al. 2008; Mitalipov and Wolf 2009). Because of their origin and pluripotency, stem cells are of major therapeutic interest in regenerative medicine as they provide a powerful source for cell replacement and tissue regeneration. This is evident in cases of damage-, disease and age-related cellular degeneration, such as spinal cord injury (Nandoe Tewarie et al. 2009; Kim and de Vellis 2009) or age-related neurodegeneration seen for example in Alzheimer's and Parkinson's disease (Daniela et al. 2007; Li et al. 2008), where stem cell therapy may become one way of replacing lost cells (Rosser et al. 2007; Ormerod et al. 2008).

However, there are several obstacles that need to be resolved before stem cell based therapies can be translated clinically. These obstacles include the unlimited proliferation potential of stem cells as well as our incomplete knowledge about the molecular machinery underlying cellular differentiation programs. Thus, a major challenge is the identification of stem cell-derived molecular determinants inherited by differentiating progenitor cells that are required for the specification of the variety of different cell types in the adult organism. Successful cell replacement and tissue regeneration is only achieved once the new cells differentiate into the desired cell type and integrate into existing cell clusters, tissues and organs. This is particularly evident for the nervous system, where the majority of cells are post-mitotic and integrated into elaborate neural circuits underlying complex behaviour. For example, a major challenge will be to induce effective functional integration of stem cell-derived neurons into existing neural circuits with the ultimate goal to restore behavioural deficits caused by progressive neurodegeneration (Lindvall and Kokaia 2006; Ormerod et al. 2008).

Equally important is the need to understand how growth and proliferation of stem cells is regulated at the molecular level in order to regenerate tissue without unwanted over-proliferation that may lead to cancer formation, but also to control undesired growth that may jeopardize final tissue and organ size. Here, regenerative medicine faces two challenges at the same time. First, stem cell proliferation needs to be restricted to a certain number of mitotic divisions until a defined and limited amount of differentiating progeny is generated. Second, and at the same time, the size of each individual differentiating cell needs to be regulated in the context of its neighbours so that a cell cluster, tissue or organ reaches an appropriate final and functional size. It is obvious from these obstacles that a solid and comprehensive understanding of the molecular mechanisms underlying stem cell proliferation and differentiation are fundamental prerequisites for the successful application of stem cells in regenerative medicine.

The majority of our current understanding comes from studies investigating asymmetric stem cell division in model organisms such as the nematode *Caenorhabditis elegans* (*C. elegans*), the insect *Drosophila melanogaster* and the laboratory mouse, *Mus musculus*. These animals are seemingly very different to humans and the ancestors of worms and flies already separated from the vertebrate lineage more than 600 Ma ago during the course of evolution (Adoutte et al. 2000;

Peterson et al. 2004). However, worms, flies and mice share several key features relevant to human stem cell biology and tissue regeneration. Whole genome sequencing revealed striking similarities in the structural composition of individual genes of *Homo sapiens*, mouse, *Drosophila* and *C. elegans*. For example, the nucleotide sequence of the gene encoding actin is almost similar in all four species, providing compelling evidence for structural conservation due to common origin (homology). Moreover, the molecules and mechanisms underlying core modules of cell biology are conserved as well: homologous genes mediate homologous mechanisms such as cyclin/cdk modules regulating the eukaryotic cell cycle (Edgar and Lehner 1996; Bähler 2005; Sánchez and Dynlacht 2005), or insulin signalling regulating metazoan cell growth (Stocker and Hafen 2000; Hietakangas and Cohen 2009). These data provide compelling evidence for a deep homology underlying cell biological mechanisms. This notion is further supported by experiments demonstrating that *Drosophila* and human genes can substitute each other in species-specific but evolutionary conserved mechanisms underlying embryonic brain development in insects and mammals (Leuzinger et al. 1998; Nagao et al. 1998; Hanks et al. 1998).

These principles of homology seem to apply to stem cell biology as well. There is mounting evidence that the mechanisms underlying asymmetric stem cell division are conserved across species. Therefore, knowledge gained in model organisms is invaluable to enhance our understanding of stem cell biology for its successful application in regenerative medicine. Experimental studies in *C. elegans*, *Drosophila* and mice have identified molecules involved in cell-intrinsic and cell-extrinsic mechanisms underlying asymmetric stem cell division which are outlined in this article.

4.2 Classifications and Definitions

Stem cells are classified by the range of commitment options and thus their lineage potential available to them (Smith 2006). *Totipotent* stem cells are sufficient to form an entire organism, whereas *pluripotent* stem cells are able to form all the body's cell lineages, including germ cells; a typical example for the latter is an embryonic stem cell (ESC). *Multipotent* stem cells can form multiple lineages that constitute an entire tissue or tissues, such as hematopoietic stem cells, whereas *unipotent* and *oligopotent* stem cells are able to form one (uni-), two or more (oligo-) lineages within a tissue.

Stem cells can continuously produce daughter cells that are either similar resulting from *symmetric* stem cell division, or they generate different daughter cells by *asymmetric* stem cell division (Fig. 4.1a). Asymmetric division leads to two distinct daughter cells from a single mitosis, usually a self-renewing stem cell, and a progenitor cell that has the capacity to differentiate. *Self-renewal* is a defining property of stem cells and the term *commitment* characterises their exit from self-renewal leading to differentiation. Self-renewal and asymmetry can be established and maintained by intrinsic and extrinsic signals.

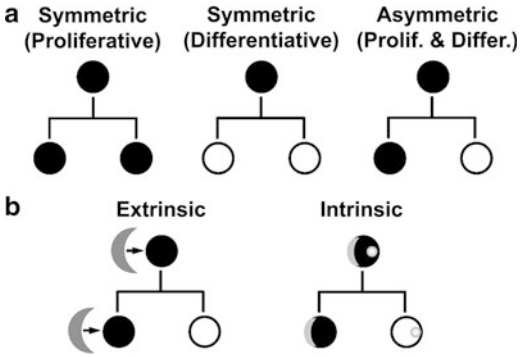


Fig. 4.1 Regulation of stem cell division. (**a**) Different modes of stem cell division. Stem cells (*filled circles*) show different modes of divisions, which can be either symmetric or asymmetric thereby regulating the number of stem cells and differentiating progeny (*open circles*) in developing and adult organisms. Symmetric, proliferative stem cell division expands the stem cell pool, whereas symmetric, differentiative stem cell division depletes the stem cell pool by generating differentiating progenitor and/or postmitotic cells. Asymmetric stem cell division can be regarded as a mixture of both proliferative and differentiative stem cell division, as it results in a self-renewing stem cell and a differentiating progenitor and/or postmitotic cells. Thus, asymmetric stem cell division maintains the stem cell pool while at the same time generates differentiating progeny. (**b**) Asymmetric stem cell division can be regulated by extrinsic or intrinsic mechanisms. Extrinsic regulation relies on asymmetric contact of the stem cell (*filled circles*) with a "niche" (*grey crescent* adjacent to stem cell) that provides support and stimuli necessary for self-renewal and to prevent differentiation. Following cell division, the cell adjacent to the niche remains a self-renewing stem cell, whereas the second daughter cell lacking contact to the niche is committed to differentiate. Intrinsic mechanisms regulate the exclusive segregation of cell fate determinants into daughter cells, with apical polarity cues (*grey crescent* within the stem cell) required for stem cell maintenance, and basal cell fate determinants (*grey circle*) required for terminal differentiation (Modified after Caussinus and Hirth 2007)

Extrinsic mechanisms are usually summarised by the term "*niche*" which characterises a cellular micro-environment that provides stimuli and support necessary to maintain self-renewal to the stem cell located adjacent to the niche (Fig. 4.1b). At the same time, the niche can generate asymmetry provided that the plane of cell division is parallel to the signals of the niche, resulting in only one daughter cell (the self-renewing stem cell) that retains contact to the niche (Fig. 4.1b). The sibling daughter cell does no longer receive niche signals, and hence can no longer maintain a self-renewing mode of division and is forced into cell cycle exit and differentiation (for review see, Li and Xie 2005; Roeder and Lorenz 2006; Martinez-Agosto et al. 2007; Mitsiadis et al. 2007; Morrison and Spradling 2008; Kuang et al. 2008; Losick et al. 2011).

Intrinsic signals refer to mechanisms and molecules acting within a dividing stem cell; they regulate the mode of division and hence the fate and commitment of its daughter cells (Fig. 4.1b). The majority of our knowledge about stem cells and asymmetric cell division come from insights into cell-intrinsic mechanisms which are outlined below.

4.3 Principles and Mechanisms

In general, four interrelated mechanisms underlie the molecular machinery regulating intrinsic asymmetric cell division: symmetry break, polarity formation, mitotic spindle orientation and segregation of cell fate determinants (see Fig. 4.2). Asymmetric stem cell division usually results in two cells that differ in fate but sometimes also in size: a self-renewing stem cell and a differentiating daughter cell.

4.3.1 Generating Asymmetry

Initially, stem cell symmetry is broken by signals from the niche or by an overlaying polarity inherited from the tissue of origin from where the stem cell derives, as in the case of epithelial cells (Fig. 4.2a). For example, in the *Drosophila* embryonic nervous system, a symmetry break already occurs in the polarised neuroectodermal epithelium from which neural stem cells, termed neuroblasts delaminate. In the *C. elegans* zygote, symmetry is broken by an actomyosin network present on the cell cortex of newly fertilized embryos. This actomysoin network drives surface contractions around the circumference which requires activity of the small GTPase Rho and its activator, the Rho guanine nucleotide exchange factor ECT-2 (for review, see Gönzy 2008). At the end of the first cell cycle the contractile cortex covers the anterior half of the embryo and the non-contractile cortex covers the posterior half, resulting in broken symmetry and initial polarity formation.

The activity of Rho GTPases is highly conserved and plays a key role in the initial steps of polarity formation in various tissues and cell types, including T-cells and epithelial cells of the lung, gut and skin (for review, see Iden and Collard 2008). Rho GTPases function as molecular switches and cycle between an active, GTP-bound state predominantly associated with membranes, and an inactive, GDP-bound state that is present in the cytoplasm. In all cases, Rho GTPases regulate and coordinate cytoskeleton remodelling, thereby providing a scaffold for symmetry break.

4.3.2 Polarity Formation

As soon as symmetry is broken, the emerging cell polarity becomes stabilized by evolutionary conserved *partitioning defective* (PAR) proteins and associated components (Fig. 4.2b). This is the case in *C. elegans* and *Drosophila*, but also for stem cells in other organisms including mammals (for review, see Schneider and Bowerman 2003; Cowan and Hyman 2004; Wodarz 2005; Suzuki and Ohno 2006; Goldstein and Macara 2007; Johnson 2009; Knoblich 2010).

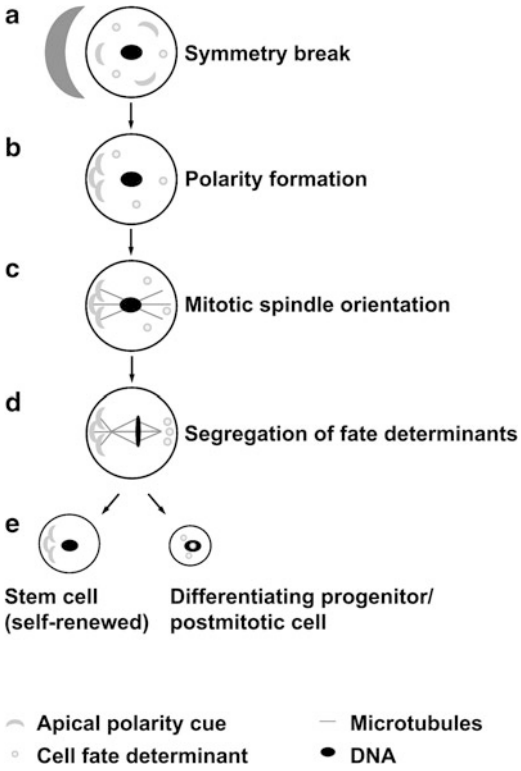


Fig. 4.2 Intrinsic asymmetric cell division. Four consecutive steps underlie intrinsic asymmetric cell division. (**a**) Symmetry break occurs in the parental stem cell either by external signals coming from the "niche" (*grey crescent* adjacent to stem cell) or from signals that have been inherited from the place of stem cell origin, such as epithelia. (**b**) Broken symmetry in turn is used to establish polarity formation, which usually involves reorganization of the actomyosin network and segregation of polarity cues along the new symmetry axis. (**c**) Subsequently, mitotic spindles are aligned perpendicular to the axis of polarity and the future cleavage plane. (**d**) Cell fate determinants are segregated along the axis of polarity and determine the fate of the future daughter cells. (**e**) Asymmetric stem cell division takes place along the axis of polarity, resulting in the unequal distribution of cell fate determinants; these in turn implement the fate of the two resulting daughter cells: a self-renewing stem cell and a differentiating progenitor/postmitotic cell (Modified after Gönzy 2008)

In *Drosophila* (see Fig. 4.3), delaminating neuroblasts inherit the PDZ domain proteins Par-3 (Bazooka) (Wodarz et al. 1999; Schober et al. 1999), Par-6 (DmPar6) (Petronczki and Knoblich 2001) and the *Drosophila* atypical protein kinase C (DaPKC) (Betschinger et al. 2003; Rolls et al. 2003; Izumi et al. 2004). Once the neuroblast has delaminated from the neuroectoderm, mitotic spindle align perpendicular to the epithelial plane (Kaltschmidt et al. 2000) and the adaptor protein Inscuteable (Insc) (Kraut et al. 1996) binds to the apical protein complex through Bazooka. Inscuteable, in turn, recruits another adaptor protein, Partner of Inscuteable

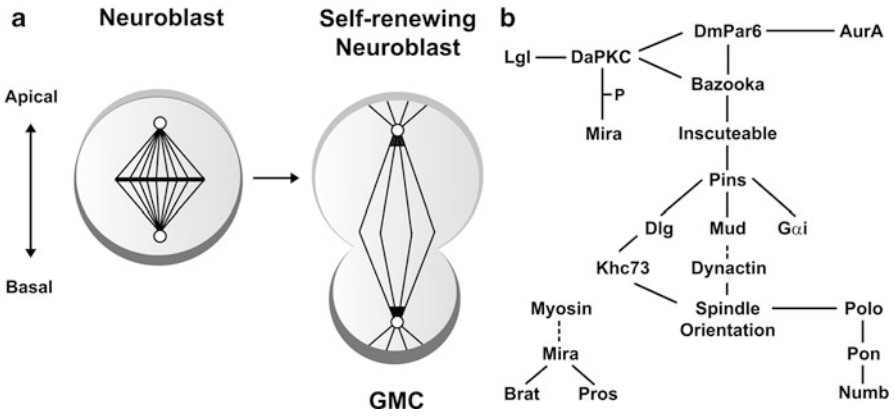


Fig. 4.3 Asymmetric stem cell division in the nervous system of *Drosophila*. (**a**) Asymmetric neuroblast division in *Drosophila*. In the developing nervous system of *Drosophila*, neural stem cells called neuroblasts divide asymmetrically along their apical-basal axis to give rise to another, self-renewing neuroblast and an intermediate progenitor cell, called ganglion mother cell (GMC). The GMC in turn exits the cell cycle and differentiates into neurons (or glial cells) by one terminal division. (**b**) Molecular machinery underlying asymmetric neuroblast division in *Drosophila*. Dividing neuroblasts are polarized along the apical-basal axis: Apical polarity cues include Lgl, DaPKC, Baz, Par6, Insc, Pins, Dlg, Mud, Gαi and AurA; spindle orientation is directed by Mud, dynactin and Khc73. Basal cell fate determinants and their adaptor proteins include Mira, Pros, Brat, Polo, Pon and Numb. During asymmetric neuroblast division, apical complex formation and basal targeting simultaneously ensure stem cell self-renewal (apical) and the formation of a differentiating GMC (basal). See text for details (Modified after Kim and Hirth 2009)

(Pins) which together bind the heterotrimeric G-protein α-subunit Gαi into the complex to form an apical crescent at late interphase/early prophase (Yu et al. 2005; Nipper et al. 2007).

Binding of Gαi to Insc enables Pins to recruit an additional protein called Mushroom body defect (Mud) (Izumi et al. 2006; Siller et al. 2006; Bowman et al. 2006) which is the *Drosophila* homolog of the microtubule and dynein binding protein NuMA (Zheng 2000; Sun and Schatten 2006). Mud is thought to interact with the astral microtubules to 'fix' one of the spindle poles on the apical cortex of the neuroblast, thus contributing to the orientation of the mitotic spindle. Pins also binds to a membrane associated guanylyl kinase protein called Discs large (Dlg), that is known to interact with Kinesin heavy chain 73 (Khc-73), localised at the plus ends of astral microtubules. These interactions polarise the complex of proteins localised at the apical cortex of *Drosophila* neuroblasts in the direction of the mitotic spindle, which aligns perpendicular to the overlying epithelial plane (Yu et al. 2005; Wang et al. 2006a; Siegrist and Doe 2007). Thus, molecules of the apical complex direct apical-basal spindle orientation in dividing neuroblasts, and thereby establish an axis of polarity along which cytokinesis takes place.

Comparable mechanisms have been found in *C. elegans* and mammals (Gönzy 2008; Siller and Doe 2009; Knoblich 2010), suggesting that interactions between

the PAR complex, heterotrimeric G-proteins and mitotic spindle orientation represent a highly conserved mechanism underlying polarity formation. As a result of polarity formation, cell fate determinants are distributed unequally along the axis of polarity, which is usually the apical-basal axis and the future axis of division (Fig. 4.2c). Importantly, as the name states, these cell fate determinants predefine the destiny of the resulting daughter cells and their unequal segregation implements proper asymmetric cell division.

4.3.3 Apical Polarity and Mitotic Spindle Orientation

As soon as an axis of polarity is established, asymmetric cell division secures the segregation cell fate determinants into only one of the resulting daughter cells, thereby regulating a binary switch between stem cell self-renewal and differentiation. This is achieved by asymmetric localisation and subsequent unequal segregation of fate determinants that promote either stem cell identity or intermediate progenitor cell identity (Fig. 4.2d). In dividing *Drosophila* neuroblasts this results in apically localised proteins being maintained in self-renewing neuroblasts, whereas basally localised proteins are segregated into differentiating progenitor cells, termed ganglion mother cells (GMCs) (for review, see Kim and Hirth 2009).

In *Drosophila* neuroblasts (Fig. 4.3a), asymmetric segregation of cell fate determinants requires key substrates including the cortically localised tumour suppressor proteins Discs large (Dlg) and Lethal (2) giant larvae (Lgl) (Ohshiro et al. 2000; Peng et al. 2000; Albertson and Doe 2003; Betschinger et al. 2003). DaPKC and Lgl are key players in the establishment and maintenance of apical polarity, thereby providing neuroblasts with the capacity to self-renew. Lgl is a cytoskeletal protein known to specify the basolateral domain and to restrict DaPKC, Bazooka, and DmPar6 to the apical cortex (Wirtz-Peitz and Knoblich 2006). Lgl does not directly influence spindle orientation and apical localisation of the PAR complex. However, phosphorylation of Lgl by DaPKC leads to Lgl inactivation, or exclusion of Lgl from the apical cortex (Betschinger et al. 2003), thereby restricting cortical recruitment of basal cell fate determinants (Fig. 4.3b).

These observations have been substantiated by mutant studies, showing that neural lineages mutant for Lgl lead to supernumerary postembryonic neuroblasts due to occasional ectopic self-renewal (Rolls et al. 2003; Lee et al. 2006a). Furthermore, overexpression of a membrane-targeted DaPKC, but not a kinase-dead mutant isoform leads to a similar phenotype, whereas a decrease in DaPKC expression reduces neuroblast numbers. Genetic interaction experiments showed that Lgl, DaPKC double mutants have normal numbers of neuroblasts and that DaPKC is fully epistatic to Lgl, suggesting that DaPKC directly promotes neuroblast self-renewal (Lee et al. 2006a).

How are DaPKC and Lgl directed to the apical cortex? A partial answer to that comes from recent data suggesting that the mitotic kinase Aurora-A (AurA) is required for the asymmetric localisation of DaPKC (Lee et al. 2006b; Wang et al.

2006b; Wirtz-Peitz et al. 2008). AurA phosphorylates DmPar6, a member of the PAR complex, which in turn prevents an interaction between DmPar6 and DaPKC (Fig. 4.3b). Subsequently, phosphorylated DaPKC can act independently of DmPar6 and is able to phosphorylate Lgl, leading to Lgl inactivation/exclusion of Lgl from the apical cortex (Betschinger et al. 2003; Lee et al. 2006a). Within the PAR complex, this sequence of events leads to the exchange of Lgl for Bazooka, which in turn enables phosphorylation of the cell fate determinant Numb and its subsequent segregation into the differentiating daughter cell, the GMC (Wirtz-Peitz et al. 2008).

These data from *Drosophila* provide a direct link between asymmetric protein localisation and mitotic spindle orientation. A linkage between apical cortex and mitotic spindle was previously identified with the Mud/NuMa protein and its role in regulating neuroblast self-renewal via proper spindle-orientation. However, previous mutant studies showed that Mud does not alter cortical polarity (Izumi et al. 2006; Siller et al. 2006; Bowman et al. 2006), whereas mutant AurA does (Lee et al. 2006b; Wang et al. 2006b; Wirtz-Peitz et al. 2008); yet, both proteins localise to the centrosomes and tissue mutant for AurA and Mud exhibits similar defects in spindle orientation (Berdnik and Knoblich 2002; Giet et al. 2002; Izumi et al. 2006; Siller et al. 2006; Bowman et al. 2006; Lee et al. 2006b; Wang et al. 2006b; Wirtz-Peitz et al. 2008). Genetic interaction data in dividing neuroblasts indicate that AurA controls mitotic spindle orientation by regulating the asymmetric localisation of Mud (Wang et al. 2006b). Moreover, AurA seems not only to act on Mud and DmPar6, but also on Notch signalling. Mutational inactivation of AurA leads to ectopic activation of Notch (Wang et al. 2006b), which in its cleaved, intracellular form is able to promote self-renewal and to suppress differentiation of neural stem cells in the larval central brain of *Drosophila*.

In addition to the Mud/NuMA and AurA axis involved in mitotic spindle orientation and asymmetric stem cell division, recent studies have identified a complementary role of the centrosome and centrioles in the regulation of stemness. These data suggest that the centrosome and centrioles of the dividing "mother stem cell" are inherited by the differentiating daughter cell (Januschke et al. 2011), whereas the newly formed centrosome and centrioles are retained by the self-renewed stem cell, a process which requires centrosomin (Cnn) function (Conduit and Raff 2010).

Taken together, data from *Drosophila* suggest that AurA acts via Mud to orient mitotic spindles required for the establishment of a proper division plane (Fig. 4.3), which is a prerequisite for the unequal segregation of cell fate determinants during neural stem cell cytokinesis (Fig. 4.3b). Simultaneously, asymmetric protein localisation is achieved, at least in part by AurA acting on DmPar6 and in turn via phosphorylation of DaPKC followed by that of Lgl. Such a dual role of AurA linking asymmetric protein localisation and mitotic spindle orientation may explain to some extend why in AurA and Mud, but also in DaPKC and Lgl mutants, the net result is the same: supernumerary neural stem cells at the expense of differentiating neurons. Moreover, recent results suggest that AurA also links Pins and Dlg to the mitotic spindle orientation pathway (Jonhston et al. 2009).

In mammals, comparable mechanisms have been observed that are involved in the establishment of apical cell polarity and mitotic spindle orientation. The mammalian cerebral cortex and retina contain multipotent neuroepithelial progenitor cells with pronounced apical/basal polarity. Their apical domain or "apical endfoot" contains a complex of Cdc42 – Par-3 – aPKC – mPar-6, similar to *Drosophila*, as well as the transmembrane protein Prominin/CD133 (for review, see Götz and Huttner 2005; Farkas and Huttner 2008; see also Chap. 12 by Huttner). Moreover, a mammalian homologue of Pins, termed LGN, can bind NuMA and links NuMa to heterotrimeric G-proteins, thereby regulating mitotic spindle orientation (Du et al. 2001; Du and Macara 2004). Mouse Inscuteable (mInsc) has been shown to play a critical role in spindle reorientation in cortical progenitors of the mouse neocortex: both loss and gain of mInsc mutations affect correct mitotic spindle positioning, which in the wildtype appears to be essential for generating the correct numbers of neurons in all cortical layers (Postiglione et al. 2011). In addition, mammalian Par-3 (mPar-3) specifies the polarity of dividing radial glial cells in the developing mouse neocortex and differentially regulates Notch signalling activity in the resulting daughter cells (Bultje et al. 2009). In mouse skin progenitor cells, recent data provide evidence that the switch from symmetric to asymmetric divisions concomitant with stratification relies on LGN, NuMA and dynactin (Dctn1) activity (Williams et al. 2011). These data suggest that at least some of the mechanisms underlying apical polarity formation and mitotic spindle alignment are evolutionary conserved and essential prerequisites for asymmetric stem cell division.

4.3.4 Basal Polarity and Cell Fate Determinants

As a result of polarity formation, two opposite sides within a stem cell are generated: an apical side and a basal side. In conjunction with polarity formation (see above), mitotic spindles are aligned and a future axis of division is established, along which apical and basal cell fate determinants are segregated during cell division (Fig. 4.2d, e). Apical cell fate determinants are involved in stem cell self-renewal, whereas basal cell fate determinants are involved in differentiation processes. This dichotomy is most obvious in the developing nervous system of *Drosophila* where self-renewal and differentiation is not only regulated in proliferating neuroblasts but also in the intermediate progenitor cells, the GMCs.

In *Drosophila*, GMCs usually are destined to exit the cell cycle by terminal, symmetric division, thereby generating the majority of neurons that constitute the adult CNS. The destiny of GMCs is determined by the exclusive inheritance of key differentiation factors such as the Notch repressor Numb (Uemura et al. 1989), the NHL-domain protein Brain tumour (Brat) (Arama et al. 2000) and the homeodomain transcription factor Prospero (Vaessin et al. 1991; Doe et al. 1991; Matsuzaki et al. 1992) (see Fig. 4.3). Basal targeting of these cell fate determinants in dividing neuroblasts is achieved via their adaptor proteins, Partner of Numb (Pon) (Lu et al. 1998) and Miranda (Shen et al. 1997; Ikeshima-Kataoka et al. 1997). Previous

experiments in *Drosophila* showed that segregation of Numb into GMCs is regulated by Pon in a cell-cycle-dependent manner, and recent data provide evidence that Polo, a key cell cycle regulator itself, is critically required for this event by direct phosphorylation of Pon (Wang et al. 2007). Accordingly, mutant polo affects the asymmetric localization of Pon, Numb and DaPKC and supernumerary neuroblast-like cells are produced at the expense of neurons. Over-expression of Numb in polo mutant lineages is able to suppress over-proliferation, indicating that Polo inhibits progenitor cell self-renewal by regulating the localization and function of Numb. As is the case for AurA, polo function therefore provides another link between cell cycle regulation and asymmetric protein localization. However, the mechanism by which Numb directly or indirectly regulates cell cycle activity and proliferation is poorly understood.

Ganglion mother cell fate is also determined by Prospero (Pros). Pros mRNA and protein is already detectable in dividing neuroblasts where it is transported via its adaptor Miranda to the basal side (Shen et al. 1998; Schuldt et al. 1998; Broadus et al. 1998; Matsuzaki et al. 1998). Cytokinesis segregates Prospero solely into the GMC where Mira degrades, thereby releasing Prospero from the cortex, which then translocates into the nucleus (Hirata et al. 1995; Spana and Doe 1995). Prospero acts as a transcription factor in the GMC nucleus, where it has a dual role. Pros inhibits cell cycle progression by repressing cell cycle regulators such as cyclin A, cyclin E and the *Drosophila* cdc25 homologue, string, as well as by activating the expression of dacapo, a cyclin-dependent kinase inhibitor, ultimately leading to terminal differentiation of the GMC into two post-mitotic neurons/or glial cells (Li and Vaessin 2000; Liu et al. 2002). Moreover, genome-wide expression profiling using *prospero* loss and gain-of function embryos as a template indicate that Prospero represses neuroblast-specific apical polarity genes like inscuteable, bazooka and DaPKC, and activates expression of neural differentiation genes such as fushi tarazu and even skipped (Choksi et al. 2006). In addition, mutant analyses provide in vivo evidence that loss of *pros* results in enlarged neuroblast lineages essentially devoid of differentiating, post-mitotic neurons (Bello et al. 2006; Lee et al. 2006c; Betschinger et al. 2006). Instead, the vast majority of cells within these mutant clones show sustained expression of stem cell markers and increased mitotic activity, eventually leading to neoplastic tumor formation (Bello et al. 2006). These data indicate that loss of *pros* causes a transformation of GMCs into stem-like cells that are unable to exit the cell cycle and continue to proliferate. Based on these experimental observations, it is reasonable to consider Prospero as a gate-keeper in regulating self-renewal and differentiation in GMCs.

Another recently identified cell fate determinant is Brain Tumor (Brat). *brat* encodes a member of the conserved NHL family of proteins (Arama et al. 2000; Reymond et al. 2001; Sardiello et al. 2008). Similar to *pros*, *brat* mutation results in over-proliferating neuroblast lineages at the expense of differentiating neurons (Bowman et al. 2008; Bello et al. 2006; Lee et al. 2006c; Betschinger et al. 2006). Brat mutant neuroblast clones show cortical mis-localisation of Miranda and the loss of nuclear *pros* (Lee et al. 2006c), suggesting that these proteins may play a

role in the same molecular pathway. This is supported by genetic experiments showing that ectopic expression of Pros can rescue the tumour formation in Brat mutants in the larval central brain (Bello et al. 2006). However, Brat localisation remains unaffected in Pros mutants, whereas in Mira mutants Brat and Pros are mislocalised.

These results indicate that Mira is essential for the asymmetric localisation of the cell fate determinants Brat and Pros. This is in line with the fact that Pros can bind to the central Pros-binding domain of Miranda (Fuerstenberg et al. 1998), and Brat binds to the coiled-coil cargo binding domain of Miranda (Betschinger et al. 2006). Moreover, the interaction between the NHL domain of Brat and the C-terminal domain of Mira (Lee et al. 2006c) appears to be essential for promoting asymmetric localisation of Pros to the GMC, where it is required for cell cycle exit and neuronal fate determination. Thus, it is conceivable that Mira and its cargo proteins Brat and Pros maybe transported across the dividing NB as a complex. But what drives basal protein targeting of adaptor proteins and their respective cell fate determinants?

Previous studies suggested that the localisation of Mira and Pros appear to be dependent on actin (Broadus and Doe 1997), as well as on motor proteins, Myosins in particular (Ohshiro et al. 2000; Petritsch et al. 2003). These studies indicated an interaction between Lgl with a plus-end directed motor, myosin II (Ohshiro et al. 2000). Subsequent experiments showed that Spaghetti Squash (Sqh), the regulatory light chain of Myosin II, is required in embryonic neuroblasts both, to organize the actin cytoskeleton, thereby enabling determinants to localize to the cortex, and to confine determinants to the basal side (Barros et al. 2003). These data suggested that Myosin II is one of the motor proteins involved in basal localisation of the cell fate determinants. In line with this, Mira was also found to physically interact with Zipper, the heavy chain of myosin II (Ohshiro et al. 2000). Thus, non-phosphorylated Lgl can negatively regulate Myosin II in embryonic NBs by directly binding to it.

In Myosin II mutant studies, cell fate determinants failed to form a basal crescent in embryonic neuroblasts (Ohshiro et al. 2000), notably Mira is mis-localised uniformly around the cortex (Erben et al. 2008). Similarly, reduced Myosin VI (Jaguar) activity in embryos, leads to a failure in basal crescent formation as well, with Mira mis-localising to the cytoplasm in patches (Petritsch et al. 2003). Myosin VI transiently accumulates in the basal cortex, partially co-localises with Mira during metaphase, and in vitro studies using *Drosophila* embryonic extracts also showed physical interaction with Mira. The distinct phenotype, mode of action, and sub-cellular localisation of Myosin II and Myosin VI suggests that they may act at consecutive steps in a single pathway to localise Mira and its cargo proteins to the basal side of dividing NBs. However, it is currently not know how exactly Mira is transported to the basal side of a dividing neural stem cell. Yet, recent experimental evidence suggests that direct phosphorylation of Mira by aPKC leads to exclusion of Mira from the apical cortex (Atwood and Prehoda 2009), which is a prerequisite for its basal targeting, and in turn the unequal segregation of cell fate determinants that are transported by Mira.

4.3.5 Cell Cycle Progression and Growth Control

These data coming from studies of the developing CNS of *Drosophila* provide compelling evidence that one strategy to regulate stem cell-self-renewal and differentiation is asymmetric segregation of cell fate determinants in a dividing cell. This is achieved, in part, by asymmetric protein localisation and related mitotic spindle orientation, thereby providing a template for unequal distribution of key regulators such as AurA, DaPKC, Numb and Pros (Fig. 4.3b). Interestingly, however, such a cascade of events does not explain why mutant stem cells continue to proliferate, thereby self-renewing for an extended period of time without progressive volume decline. This is particularly evident in the case of continued proliferation in *pros* mutant neuroblast clones in *Drosophila*. There, continued cell division cycles appear to be accompanied by compensatory cell growth. Thus, *pros* mutant cells display sustained symmetric divisions without shrinkage in cell size (Bello et al. 2006), a phenomenon that is usually accompanied with neuroblast division in the embryonic CNS. Thus, in *pros* mutant clones, a constant cell size appears to be maintained over many rounds of self-renewing divisions, indicating that Pros may also act as a transcriptional repressor on genes involved in growth control. However, genome-wide expression profiling did not identify growth control genes as potential targets of *pros*, maybe because embryos had been used as a template (Choksi et al. 2006). A possible link between asymmetric protein localisation, cell cycle progression and growth control may be provided by Brat.

Previous studies in *Drosophila* had shown that *brat* is a translational repressor (Sonoda and Wharton 2001) which also functions in the regulation of cell growth and ribosomal RNA synthesis (Frank et al. 2002). Growth and proliferation of *brat* mutant cells might be perpetuated by dis-inhibited dMyc activity (Betschinger et al. 2006), a transcription factor regulating cell growth and proliferation (Eilers and Eisenman 2008). The available data however suggest that Brat activity regulates a large number of direct and indirect targets involved in cell cycle progression and growth control. This notion is supported by genome-wide expression studies using adult wildtype and *brat* mutant brain tissue as a template (Loop et al. 2004). These studies identified several potential target genes of Brat, most prominent among them genes involved in cell cycle regulation and translation control, as well as RNA binding/processing, all being up-regulated in *brat* mutant tissue (Loop et al. 2004). In addition, *brat* gain of function can inhibit cell growth and ribosomal RNA accumulation, and slowdown cell division cycles (Frank et al. 2002). Considering its mutant lineage phenotype, these data suggest that *brat* may inhibit cell growth by limiting the rate of ribosome biogenesis and protein synthesis.

Comparable data have been found in *C. elegans* where Brat homologues regulate PAR protein-dependent polarity and asymmetric cell division (Hyenne et al. 2008). In addition, homologues of Brat have been identified in mammals where they are also involved in progenitor cell proliferation control. Recent genetic evidence in mice suggests that the Brat homolog TRIM32 can bind Ago1, a protein involved in microRNA processing. TRIM32 functions both by degrading c-Myc as well as by activating certain microRNAs, among them the stem cell regulator Let-7a (Gangaraju

and Lin 2009). TRIM32 activity thereby suppresses self-renewal in dividing cortical progenitor cells, and induces neuronal differentiation (Schwamborn et al. 2009). These findings indicate that Brat/TRIM-NHL proteins regulate self-renewal and differentiation of stem/progenitor cells by modulating microRNA activity as well as ribosome biogenesis and protein synthesis.

These data also suggest that deregulated stem/progenitor cell division can lead to uncontrolled cell growth and tumor formation (Caussinus and Hirth 2007). Indeed, recent experimental evidence suggests that so-called cancer stem cells drive the growth and metastasis of human tumors and cancer stem cells have already been identified in leukemia, and in solid tumors of the breast and brain (for review, see Reya et al. 2001; Pardal et al. 2003; Al-Hajj and Clarke 2004; Fomchenko and Holland 2005; Stiles and Rowitch 2008; Visvader and Lindeman 2008; Schatton et al. 2009). Moreover, inappropriate activation of the WNT, sonic hedgehog (SHH), Notch, PTEN, and BMI1 pathways have all been shown to promote the self-renewal of somatic stem cells, and their dysregulation can lead to neoplastic tissue formation (for review, see Pardal et al. 2003; Jiang and Hui 2008).

Based on these observations, it is conceivable that similar to the situation in *Drosophila*, the machinery promoting asymmetric cell division may play an evolutionary conserved role in cell cycle control and tumor suppression. Indeed, mammalian homologues of Baz, Par6, DaPKC, Lgl, Numb and Brat have been shown to regulate asymmetric cell fate determination and tumor suppression. Thus, mammalian aPKC, Par3, and LGN are involved in asymmetric division of basal epidermal progenitor cells of the skin and their dysregulation can lead to skin cancer (Lechler and Fuchs 2005). The Brat homologue TRIM3 has been identified as a candidate brain tumor suppressor gene (Boulay et al. 2009), indicating that Brat/TRIM-NHL proteins act in a conserved genetic pathway regulating stem/progenitor cell self-renewal and differentiation. Moreover, there is evidence for the asymmetric segregation of vertebrate NUMB homologues (Wodarz and Huttner 2003) that seem to act as asymmetric cell fate determinants. Double knockouts of Numb and Numb-like in the mouse dorsal forebrain have been found to lead to impaired neuronal differentiation, hyper-proliferation of neural progenitors, and delayed cell-cycle exit (Petersen et al. 2002, 2004; Li et al. 2003). In addition, loss of Lgl1/Mlgl/Hugl, one of the two Lgl homologues in mice, results in failure to asymmetrically localize the fate determinant Numb and leads to severe brain dysplasia as neural progenitor cells fail to exit the cell cycle (Klezovitch et al. 2004). Reciprocally, a well-characterized human tumor suppressor, the kinase Lkb1, whose loss-of-function phenotype results in Peutz-Jeghers syndrome, regulates cell polarity in worms, flies and humans and might be involved in asymmetric cell division as well (Marignani 2005). In addition, recent data provide compelling evidence that also mammalian homologues of Notch, NuMa and dynactin (Williams et al. 2011) as well as Inscuteable (Postiglione et al. 2011) contribute to maintain a proper balance between neuronal proliferation and differentiation in the developing mouse neocortex. Thus, similar to the situation in *Drosophila*, asymmetric cell division in mammals appears to be involved in the regulation of stem and progenitor cell self-renewal, and the regulation of cell cycle progression and growth control.

4.4 Conclusions and Perspectives

Studies using model organisms, including the *C. elegans*, *Drosophila melanogaster*, and mice have revealed insights into the molecular mechanisms underlying asymmetric stem cell division. These studies identified key essential, consecutive steps of asymmetric cell division that are characterised by symmetry break, polarity formation, mitotic spindle orientation and segregation of cell fate determinants; these processes are mediated by evolutionary conserved molecules, including Aurora-A, aPKC, Mud/NuMa, Lgl, Numb and Brat/TRIM-NHL proteins. Asymmetric stem cell division lies at the interface of stem cell self-renewal and differentiation and therefore regulates the number and identity of differentiating progeny. Therefore, asymmetric cell division is of major therapeutic interest in regenerative medicine as asymmetrically dividing stem cells provide a powerful source for targeted cell replacement and tissue regeneration. For therapeutic applications, it will now be important to determine further details of the machinery involved, in order to be able to manipulate asymmetric stem cell division in vitro for the unlimited generation of differentiated cells at will. Several key questions need to be addressed and answered in order to achieve these goals. These include elucidation of the mechanisms and molecules that define and maintain stemness; to identify molecules that regulate the binary switch between self-renewal and differentiation; to determine the mechanisms that direct cell type specific differentiation; and to determine ways how an in vitro generated cell can integrate into an existing cellular context while remaining differentiated. Elucidating the molecular mechanisms regulating asymmetric stem cell division will significantly contribute to the successful application of stem cells in regenerative medicine.

Acknowledgements Work in the Hirth laboratory is supported by grants from the UK Medical Research Council (G070149), the Royal Society (Hirth/2007/R2), the Parkinson's Disease Society (G-0714), the Motor Neurone Disease Association (Hirth/Oct07/6233), and the Fondation Thierry Latran (Hirth/DrosALS).

References

Adoutte A, Balavoine G, Lartillot N, Lespinet O, Prud'homme B, de Rosa R (2000) The new animal phylogeny: reliability and implications. Proc Natl Acad Sci USA 97:4453–4465

Albertson R, Doe CQ (2003) Dlg, Scrib and Lgl regulate neuroblast cell size and mitotic spindle asymmetry. Nat Cell Biol 5:166–170

Al-Hajj M, Clarke MF (2004) Self-renewal and solid tumor stem cells. Oncogene 23:7274–7282

Arama E, Dickman D, Kinchie Z, Shearn A, Lev Z (2000) Mutations in the beta-propeller domain of the Drosophila brain tumor (brat) protein induce neoplasm in the larval brain. Oncogene 19:3706–3716

Atwood SX, Prehoda KE (2009) aPKC phosphorylates Miranda to polarize fate determinants during neuroblast asymmetric cell division. Curr Biol 19:723–729

Bähler J (2005) Cell-cycle control of gene expression in budding and fission yeast. Annu Rev Genet 39:69–94

Barros CS, Phelps CB, Brand AH (2003) Drosophila nonmuscle Myosin II promotes the asymmetric segregation of cell fate determinants by cotical exclusion rather than active transport. Dev Cell 5:829–840

Bello BC, Reichert H, Hirth F (2006) The brain tumour gene negatively regulates neural progenitor cell proliferation in the larval central brain of Drosophila. Development 133:2639–2648

Berdnik D, Knoblich JA (2002) Drosophila Aurora-A is required for centrosome maturation and actin-dependent asymmetric protein localization during mitosis. Curr Biol 12:640–647

Betschinger J, Mechtler K, Knoblich JA (2003) The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. Nature 422:326–330

Betschinger J, Mechtler K, Knoblich JA (2006) Asymmetric segregation of the tumour suppressor Brat regulates self-renewal in drosophila neural stem cells. Cell 124:1241–1253

Boulay JL, Stiefel U, Taylor E, Dolder B, Merlo A, Hirth F (2009) Loss of heterozygosity of TRIM3 in malignant gliomas. BMC Cancer 9:71

Bowman SK, Neumuller RA, Novatchkova M, Du Q, Knoblich JA (2006) The Drosophila NuMa homolog Mud regulates spindle orientation in asymmetric cell division. Dev Cell 10:731–742

Bowman SK, Rolland V, Betschinger J, Kinsey KA, Emery G, Knoblich JA (2008) The tumor suppressors Brat and Numb regulate transit-amplifying neuroblast lineages in Drosophila. Dev Cell 14:535–546

Broadus J, Doe CQ (1997) Extrinsic cues, intrinsic cues and microfilaments regulate asymmetric protein localization in Drosophila neuroblasts. Curr Biol 7:827–835

Broadus J, Fuerstenberg S, Doe CQ (1998) Staufen-dependent localization of prospero mRNA contributes to neuroblast daughter-cell fate. Nature 391:792–795

Bultje RS, Castaneda-Castellanos DR, Jan LY, Jan YN, Kriegstein AR, Shi SH (2009) Mammalian Par3 regulates progenitor cell asymmetric division via notch signaling in the developing neocortex. Neuron 63:189–202

Caussinus E, Hirth F (2007) Asymmetric stem cell division in development and cancer. Prog Mol Subcell Biol 45:205–225

Choksi SP, Southall TD, Bossing T, Edoff K, De Wit E, Fischer BE, Van Steensel B, Miclem G, Brand AH (2006) Prospero acts as a binary switch between self renewal and differentiation in Drosophila neural stem cells. Dev Cell 11:775–789

Conduit PT, Raff JW (2010) Cnn dynamics drive centrosome size asymmetry to ensure daughter centriole retention in Drosophila neuroblasts. Curr Biol 20:2187–2292

Cowan CR, Hyman AA (2004) Asymmetric cell division in *C. elegans*: cortical polarity and spindle positioning. Annu Rev Cell Dev Biol 20:427–453

Daniela F, Vescovi AL, Bottai D (2007) The stem cells as a potential treatment for neurodegeneration. Methods Mol Biol 399:199–213

Doe CQ, Chu-LaGraff Q, Wright DM, Scott MP (1991) The prospero gene specifies cell fates in the Drosophila central nervous system. Cell 65:451–464

Du Q, Macara IG (2004) Mammalian Pins is a conformational switch that links NuMA to heterotrimeric G proteins. Cell 119:503–516

Du Q, Stukenberg PT, Macara IG (2001) A mammalian partner of inscuteable binds NuMA and regulates mitotic spindle organization. Nat Cell Biol 3:1069–7105

Eckfeldt CE, Mendenhall EM, Verfaillie CM (2005) The molecular repertoire of the 'almighty' stem cell. Nat Rev Mol Cell Biol 6:726–737

Edgar BA, Lehner CF (1996) Developmental control of cell cycle regulators: a fly's perspective. Science 274:1646–1652

Eilers M, Eisenman RN (2008) Myc's broad reach. Genes Dev 22:2755–2766

Erben V, Waldhuber M, Langer D, Fetka I, Jansen RP, Petritsch C (2008) Asymmetric localisation of the adaptor protein Miranda in neuroblasts is achieved by the diffusion and sequential interaction of myosin II and VI. J Cell Sci 121:1403–1414

Farkas LM, Huttner WB (2008) The cell biology of neural stem and progenitor cells and its significance for their proliferation versus differentiation during mammalian brain development. Curr Opin Cell Biol 20:707–715

Fomchenko EI, Holland EC (2005) Stem cells and brain cancer. Exp Cell Res 306:323–329
Frank DJ, Edgar BA, Roth MB (2002) The Drosophila melanogaster gene brain tumor negatively regulates cell growth and ribosomal RNA synthesis. Development 129:399–407
Fuerstenberg S, Peng CY, Alvarez-Ortiz P, Hor T, Doe CQ (1998) Identification of Miranda protein domains regulating asymmetric cortical localisation, cargo binding, and cortical release. Mol Cell Neurosci 12:325–339
Gangaraju VK, Lin H (2009) MicroRNAs: key regulators of stem cells. Nat Rev Mol Cell Biol 10:116–125
Giet R, McLean D, Descamps S, Lee MJ, Raff JW, Prigent C, Glover DM (2002) Drosophila Aurora A kinase is required to localize D-TACC to centrosomes and to regulate astral microtubules. J Cell Biol 156:437–451
Goldstein B, Macara IG (2007) The PAR proteins: fundamental players in animal cell polarization. Dev Cell 13:609–622
Gönzy P (2008) Mechanisms of asymmetric cell division: flies and worms pave the way. Nat Rev Mol Cell Biol 9:355–366
Götz M, Huttner WB (2005) The cell biology of neurogenesis. Nat Rev Mol Cell Biol 6:777–788
Hanks MC, Loomis CA, Harris E, Tong CX, Anson-Cartwright L, Auerbach A, Joyner A (1998) Drosophila engrailed can substitute for mouse Engrailed1 function in mid-hindbrain, but not limb development. Development 125:4521–4530
Hietakangas V, Cohen SM (2009) Regulation of tissue growth through nutrient sensing. Annu Rev Genet 43:389–410
Hirata J, Nakagoshi H, Nabeshima Y, Matsuzaki F (1995) Asymmetric segregation of the homeodomain protein Prospero during Drosophila development. Nature 377:627–630
Hyenne V, Desrosiers M, Labbé JC (2008) *C. elegans* Brat homologs regulate PAR protein-dependent polarity and asymmetric cell division. Dev Biol 321(2):368–378
Iden S, Collard JG (2008) Crosstalk between small GTPases and polarity proteins in cell polarization. Nat Rev Mol Cell Biol 9:846–859
Ikeshima-Kataoka H, Skeath JB, Nabeshima Y, Doe CQ, Matsuzaki F (1997) Miranda directs Prospero to a daughter cell during Drosophila asymmetric divisions. Nature 390:625–629
Izumi Y, Ohta N, Itoh-Furuya A, Fuse N, Matsuzaki F (2004) Differential functions of G protein and Baz-aPKC signalling pathways in *Drosophila* neuroblast asymmetric division. J Cell Biol 164:729–738
Izumi Y, Ohta N, Hisata K, Raabe T, Matsuzaki F (2006) *Drosophila* Pins-binding protein Mud regulates spindle-polarity coupling and centrosome organization. Nat Cell Biol 8:586–593
Januschke J, Llamazares S, Reina J, Gonzalez C (2011) Drosophila neuroblasts retain the daughter centrosome. Nat Commun 2:243
Jiang J, Hui CC (2008) Hedgehog signaling in development and cancer. Dev Cell 15:801–812
Johnson MH (2009) From mouse egg to mouse embryo: polarities, axes, and tissues. Annu Rev Cell Dev Biol 25:483–512
Jonhston CA, Hirono K, Prehoda KE, Doe CQ (2009) Identification of an Aurora-A/Pins^{LINKER}/Dlg spindle orientation pathway using induced cell polarity in S2 cells. Cell 138:1150–1163
Kaltschmidt JA, Davidson C, Brown NH, Brand AH (2000) Rotation and asymmetry of the mitotic spindle direct asymmetric cell division in the developing central nervous system. Nat Cell Biol 2:7–10
Kim SU, de Vellis J (2009) Stem cell-based cell therapy in neurological diseases: a review. J Neurosci Res 87:2183–2200
Kim DW, Hirth F (2009) Genetic mechanisms regulating stem cell self-renewal and differentiation in the central nervous system of Drosophila. Cell Adhes Migr 3:1–10
Klezovitch O, Fernandez TE, Tapscott SJ, Vasioukhin V (2004) Loss of cell polarity causes severe brain dysplasia in lgl1 knockout mice. Genes Dev 18:559–571
Knoblich JA (2010) Asymmetric cell division: recent developments and their implications for tumour biology. Nat Rev Mol Cell Biol 11:849–860
Kraut R, Chia W, Jan LY, Jan YN, Knoblich JA (1996) Role of inscuteable in orienting asymmetric cell division in Drosophila. Nature 383:50–55

Kuang S, Gillespie MA, Rudnicki MA (2008) Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell 2:22–31

Lechler T, Fuchs E (2005) Asymmetric cell divisions promote stratification and differentiation of mammalian skin. Nature 437:275–280

Lee CY, Robinson KJ, Doe CQ (2006a) Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. Nature 439:594–598

Lee CY, Andersen RO, Cabernard C, Manning L, Tran KD, Lanskey MJ, Bashirullah A, Doe CQ (2006b) Drosophila Aurora-A kinase inhibits neuroblast self-renewal by regulating aPKC/Numb cortical polarity and spindle orientation. Genes Dev 20:3464–3474

Lee CY, Wilkinson BD, Siegrist SE, Wharton RP, Doe CQ (2006c) Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self renewal. Dev Cell 10:441–449

Leuzinger S, Hirth F, Gerlich D, Acampora D, Simeone A, Gehring WJ, Finkelstein R, Furukubo-Tokunaga K, Reichert H (1998) Equivalence of the fly orthodenticle gene and the human OTX genes in embryonic brain development of Drosophila. Development 125:1703–1710

Li L, Vaessin H (2000) Pan-neural Prospero terminates cell proliferation during Drosophila neurogenesis. Genes Dev 14:147–151

Li L, Xie T (2005) Stem cell niche: structure and function. Annu Rev Cell Dev Biol 21:605–631

Li HS, Wang D, Shen Q, Schonemann MD, Gorski JA, Jones KR, Temple S, Jan LY, Jan YN (2003) Inactivation of numb and numblike in embryonic dorsal forebrain impairs neurogenesis and disrupts cortical morphogenesis. Neuron 40:1105–1118

Li JY, Christophersen NS, Hall V, Soulet D, Brundin P (2008) Critical issues of clinical human embryonic stem cell therapy for brain repair. Trends Neurosci 31:146–153

Liu TH, Li L, Vaessin H (2002) Transcription of the Drosophila CKI gene dacapo is regulated by a modular array of cis-regulatory sequences. Mech Dev 112:25–36

Lindvall O, Kokaia Z (2006) Stem cells for the treatment of neurological disorders. Nature 441:1094–1096

Loop T, Leemans R, Stiefel U, Hermida L, Egger B, Xie F, Primig M, Certa U, Fischbach KF, Reichert H, Hirth F (2004) Transcriptional signature of an adult brain tumour in *Drosophila*. BMC Genomics 5:24

Losick VP, Morris LX, Fox DT, Spradling A (2011) Drosophila stem cell niches: a decade of discovery suggests a unified view of stem cell regulation. Dev Cell 21:159–171

Lu B, Rothenberg M, Jan LY, Jan YN (1998) Partner of Numb co-localises with Numb during mitosis and directs Numb asymmetric localisation in Drosophila neural and muscle progenitors. Cell 95:225–235

Marignani PA (2005) LKB1, the multitasking tumour suppressor kinase. J Clin Pathol 58:15–19

Martinez-Agosto JA, Mikkola HK, Hartenstein V, Banerjee U (2007) The hematopoietic stem cell and its niche: a comparative view. Genes Dev 21:3044–3060

Matsuzaki F, Koizumi K, Hama C, Yoshioka T, Nabeshima Y (1992) Cloning of the Drosophila prospero gene and its expression in ganglion mother cells. Biochem Biophys Res Commun 182:1326–1332

Matsuzaki F, Ohshiro T, Ikeshima-Kataoka H, Izumi H (1998) Miranda localises staufen and prospero asymmetrically in mitotic neuroblasts and epithelial cells in early Drosophila embryogenesis. Development 125:4089–4098

Metallo CM, Azarin SM, Ji L, de Pablo JJ, Palecek SP (2008) Engineering tissue from human embryonic stem cells. J Cell Mol Med 12:709–729

Mitalipov S, Wolf D (2009) Totipotency, pluripotency and nuclear reprogramming. Adv Biochem Eng Biotechnol 114:185–199

Mitsiadis TA, Barrandon O, Rochat A, Barrandon Y, De Bari C (2007) Stem cell niches in mammals. Exp Cell Res 313:3377–3385

Morrison SJ, Spradling AC (2008) Stem cell and niches: mechanisms that promote stem cell maintenance throughout life. Cell 132:598–611

Murry CE, Keller G (2008) Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell 132:661–680

Nagao T, Leuzinger S, Acampora D, Simeone A, Finkelstein R, Reichert H, Furukubo-Tokunaga K (1998) Developmental rescue of Drosophila cephalic defects by the human Otx genes. Proc Natl Acad Sci USA 95:3737–3742

Nandoe Tewarie RS, Hurtado A, Bartels RH, Grotenhuis A, Oudega M (2009) Stem cell-based therapies for spinal cord injury. J Spinal Cord Med 32:105–114
Nipper RW, Siller KH, Smith NR, Doe CQ, Prehoda KE (2007) Galphai generates multiple Pins activation states to link cortical polarity and spindle orientation in Drosophila neuroblasts. Proc Natl Acad Sci USA 104:14306–14311
Ormerod BK, Palmer TD, Caldwell MA (2008) Neurodegeneration and cell replacement. Philos Trans R Soc B 363:153–170
Ohshiro T, Yagami T, Zhang C, Matsuzaki F (2000) Role of cortical tumour-suppressor proteins in asymmetric division of Drosophila neuroblast. Nature 408:596–600
Pardal R, Clarke MF, Morrison SJ (2003) Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3:895–902
Peng CY, Manning L, Albertson R, Doe CQ (2000) The tumour-suppressor genes lgl and dlg regulate basal protein targeting in Drosophila neuroblasts. Nature 408:596–600
Petersen PH, Zhou K, Hwang JK, Jan YN, Zhong W (2002) Progenitor cell maintenance requires numb and numblike during mouse neurogenesis. Nature 419:929–934
Petersen PH, Zhou K, Krauss S, Zhong W (2004) Continuing role for mouse Numb and Numbl in maintaining progenitor cells during cortical neurogenesis. Nat Neurosci 7:803–811
Peterson KJ, Lyons JB, Nowak KS, Takacs CM, Wargo MJ, McPeek MA (2004) Estimating metazoan divergence times with a molecular clock. Proc Natl Acad Sci USA 101:6536–6541
Petritsch C, Tavosanis G, Turck CW, Jan LY, Jan YN (2003) The Drosophila myosin VI jaguar is required for basal protein targeting and correct spindle orientation in mitotic neuroblasts. Dev Cell 4:273–281
Petronczki M, Knoblich JA (2001) DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblast in Drosophila. Nat Cell Biol 3:43–49
Postiglione MP, Jüschke C, Xie Y, Haas GA, Charalambous C, Knoblich JA (2011) Mouse inscuteable induces apical-Basal spindle orientation to facilitate intermediate progenitor generation in the developing neocortex. Neuron 72:269–284
Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105–111
Reymond A, Meroni G, Fantozzi A, Merla G, Cairo S, Luzi L, Riganelli D, Zanaria E, Messali S, Cainarca S, Guffanti A, Minucci S, Pelicci PG, Ballabio A (2001) The tripartite motif family identifies cell compartments. EMBO J 20:2140–2151
Roeder I, Lorenz R (2006) Asymmetry of stem cell fate and the potential impact of the niche: observations, simulations, and interpretations. Stem Cell Rev 2:171–180
Rolls MM, Albertson R, Shih HP, Lee CY, Doe CQ (2003) Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. J Cell Biol 163:1089–1098
Rosser AE, Zietlow R, Dunnett SB (2007) Stem cell transplantation for neurodegenerative diseases. Curr Opin Neurol 20:688–692
Sánchez I, Dynlacht BD (2005) New insights into cyclins, CDKs, and cell cycle control. Semin Cell Dev Biol 16:311–321
Sardiello M, Cairo S, Fontanella B, Ballabio A, Meroni G (2008) Genomic analysis of the TRIM family reveals two groups of genes with distinct evolutionary properties. BMC Evol Biol 8:225
Schatton T, Frank NY, Frank MH (2009) Identification and targeting of cancer stem cells. Bioessays 31:1038–1049
Schneider SQ, Bowerman B (2003) Cell polarity and the cytoskeleton in the Caenorhabditis elegans zygote. Annu Rev Genet 37:221–249
Schober M, Schaefer M, Knoblich JA (1999) Bazooka recruits inscuteable to orient asymmetric cell divisions in Drosophila neuroblasts. Nature 402:548–551
Schuldt AJ, Adams JH, Davidson CM, Micklem DR, Haseloff J, St Johnston D, Brand AH (1998) Miranda mediates asymmetric protein and RNA localization in the developing nervous system. Genes Dev 12:1847–1857
Schwamborn JC, Berezikov E, Knoblich JA (2009) The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. Cell 136:913–925

Shen CP, Jan LY, Jan YN (1997) Miranda is required for the asymmetric localization of Prospero during mitosis in Drosophila. Cell 90:449–458
Shen CP, Knoblich JA, Chan YM, Jiang MM, Jan LY, Jan YN (1998) Miranda as a multidomain adapter linking apically localized inscuteable and basally localized Staufen and Prospero during asymmetric cell division in Drosophila. Genes Dev 12:1837–1846
Siegrist SE, Doe CQ (2007) Microtubule-induced cortical cell polarity. Genes Dev 21:483–496
Siller KH, Doe CQ (2009) Spindle orientation during asymmetric cell division. Nat Cell Biol 11:365–374
Siller KH, Cabernard C, Doe CQ (2006) The NuMA-related Mud protein binds Pins and regulates spindle orientation in Drosophila neuroblasts. Nat Cell Biol 8:594–600
Slack JM (2008) Origin of stem cells in organogenesis. Science 322:1498–1501
Smith A (2006) A glossary for stem cell biology. Nature 441:1060
Sonoda J, Wharton RP (2001) *Drosophila* brain tumour is a translational repressor. Genes Dev 15:762–773
Spana EP, Doe CQ (1995) The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in Drosophila. Development 121:3187–3195
Stiles CD, Rowitch DH (2008) Glioma stem cells: a midterm exam. Neuron 58:832–846
Stocker H, Hafen E (2000) Genetic control of cell size. Curr Opin Genet Dev 10:529–535
Sun QY, Schatten H (2006) Role of NuMA in vertebrate cells: review of an intriguing multifunctional protein. Front Biosci 11:1137–1146
Suzuki A, Ohno S (2006) The PAR-aPKC system: lessons in polarity. J Cell Sci 119:979–987
Uemura T, Shepherd S, Ackerman L, Jan LY, Jan YN (1989) Numb, a gene required in determination of cell fate during sensory organ formation in Drosophila embryos. Cell 58:349–360
Vaessin H, Grell E, Wolff E, Bier E, Jan LY, Jan YN (1991) Prospero is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in Drosophila. Cell 67:941–953
Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8:755–768
Wang H, Cai Y, Chia W, Yang X (2006a) Drosophila homologs of mammalian TNF/TNFR-related molecules regulate segregation of Miranda/Prospero in neuroblasts. EMBO J 25:5783–5793
Wang H, Somers GW, Bashirullah A, Heberlein U, Yu F, Chia W (2006b) Aurora-A acts as a tumour suppressor and regulates self-renewal of Drosophila neuroblasts. Genes Dev 20:3453–3463
Wang H, Ouyang Y, Somers WG, Chia W, Lu B (2007) Polo inhibits progenitor self-renewal and regulates Numb asymmetry by phosphorylating Pon. Nature 449:96–100
Williams SE, Beronja S, Pasolli HA, Fuchs E (2011) Asymmetric cell divisions promote Notch-dependent epidermal differentiation. Nature 470:353–358
Wirtz-Peitz F, Knoblich JA (2006) Lethal giant larvae take on a life of their own. Trends Cell Biol 16:234–241
Wirtz-Peitz F, Nishimura T, Knoblich JA (2008) Linking cell cycle to asymmetric division: Aurora-A phosphorylates the Par complex to regulate Numb localization. Cell 135:161–173
Wodarz A (2005) Molecular control of cell polarity and asymmetric cell division in Drosophila neuroblasts. Curr Opin Cell Biol 17:475–481
Wodarz A, Huttner WB (2003) Asymmetric cell division during neurogenesis of *Drosophila* and vertebrates. Mech Dev 120:1297–1309
Wodarz A, Ramrath A, Kuchinke U, Knust E (1999) Bazooka provides an apical cue for inscuteable localisation in Drosophila neuroblasts. Nature 402:544–547
Yu F, Wang H, Qian H, Kaushik R, Bownes M, Yang X, Chia W (2005) Locomotion defects, together with Pins, regulates heterotrimeric G-protein signalling during Drosophila neuroblast asymmetric divisions. Genes Dev 19:1341–1353
Zheng C (2000) NuMA: a nuclear protein involved in mitotic centrosome function. Microsc Res Tech 49:467–477

Chapter 5
Stem Cells in the Developing and Adult Nervous System

Fumitaka Osakada and Masayo Takahashi

Abstract The fertilized egg is a totipotent stem cell that can produce all cell types of the organism, including the embryonic and the extraembryonic tissues. As development proceeds, cells lose their capacity to proliferate and differentiate into different cell types, and gain specialization. However, advances in stem cell biology have provided new insights into development and regenerative medicine. For example, neural stem/progenitor cells have been found to exist not only during embryonic development, but also in the adult nervous system of mammals. Newborn neurons in the adult brain integrate into pre-existing neural circuits and exhibit functional similarity to neurons born during development. Moreover, although development of an organism proceeds irreversibly from embryo to adult with cells differentiating progressively toward specialized cell types, somatic cells can be artificially reprogrammed to adopt a different cell fate, as exemplified by induced pluripotent stem cells (iPS cells) and induced neuronal cells (iN cells). Here, we summarize the current views of stem cell biology during embryogenesis and adult neurogenesis. We also discuss therapeutic potential of stem cells, focusing on retinal development and regeneration.

F. Osakada, Ph.D. (✉)
Systems Neurobiology Laboratory, The Salk Institute for Biological Studies,
10010 North Torrey Pines Road, La Jolla, CA 92037, USA
e-mail: fosakada@salk.edu

M. Takahashi, M.D., Ph.D.
Laboratory for Retinal Regeneration, Center for Developmental Biology, RIKEN,
2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_5,

5.1 Stem Cell Hierarchy

Stem cells and progenitor cells possess the remarkable ability to give rise to multiple cell types while maintaining their capacity to self-renew, or produce more stem or progenitor cells (Gotz and Huttner 2005; Morrison and Kimble 2006). This is accomplished in part through different types of cell division. Symmetric divisions of stem cells increase the stem cell population, whereas asymmetric divisions produce another stem cell and a progenitor cell that is more restricted in its differentiation capacity (Fig. 5.1a, b). In contrast, symmetric divisions of progenitor cells, which are generally thought to be in a slightly more differentiated state than stem cells, generate two daughter cells that are identical to each other but different from the mother cell (Fig. 5.1c). Asymmetric divisions of progenitor cells generate two types of differentiated daughter cells that are different from the mother cell (Fig. 5.1d).

During development, cells differ in their ability to differentiate into other cell types. The fertilized egg is totipotent, meaning that it can develop into every cell type in an organism (Fig. 5.2). However, with successive divisions, cells in the embryo lose their potential and progressively become more and more specialized. For example, the pluripotent inner cell mass (ICM) of the mammalian blastocyst stage embryo gives rise to cells in all three embryonic germ layers, but not to the extraembryonic trophoblast lineage (Niwa 2007). Embryonic stem (ES) cells, which are cell lines derived from the ICM that can grow indefinitely *in vitro* (Evans and

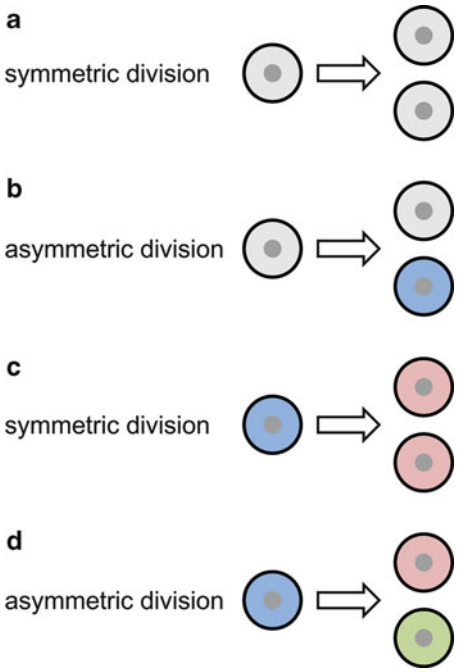


Fig. 5.1 Symmetric and asymmetric divisions of stem cells. (**a**) Symmetric division of stem cells generates two identical copies of the mother cell. (**b**) Asymmetric division of stem cells produces one new stem cell that is identical to the mother cell and another cell that is destined to differentiate along a particular lineage. (**c**) Symmetric division of progenitor cells generates two daughter cells that are identical to each other but different from the mother cell. (**d**) Asymmetric division of progenitor cells generates two types of differentiated daughter cells. *Gray*: stem cell, *Blue*: progenitor cell, *Red* or *green*: differentiated cell

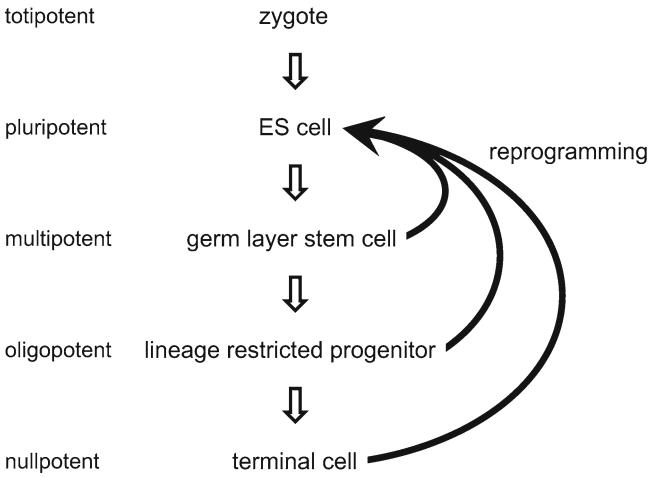


Fig. 5.2 Stem cell hierarchy during development and reprogramming. As development proceeds, cells lose their capacity to proliferate and differentiate into different cell types, and gain specialization. Normally, cells undergo progressive and irreversible differentiation into specialized cell types (*white arrow*). However, differentiated cells can be artificially reprogrammed and returned to the naive state of pluripotency found in the early embryo, as exemplified by iPS cells (*black arrow*)

Kaufman 1981), are also pluripotent. Within each germ layer, multipotent stem cells or progenitor cells are able to develop into several different cell types, but are more restricted in potential than ICM or ES cells. For example, a cell in the ectoderm gives rise to neural stem cells or progenitor cells, which divide to produce three types of terminally differentiated cells: neurons, astrocytes, and oligodendrocytes. Within tissues and organs, bipotent cells are able to develop into two cell types. Finally, when a cell's potential has been completely restricted, it is committed to undergo terminal differentiation into only a single cell type.

Although the development of an embryo into an adult normally entails progressive and irreversible differentiation of cells into their final, specialized fates, adult somatic cells can be artificially reprogrammed and returned to the naive state of pluripotency found in the early embryo (Fig. 5.2). Over 50 years ago, Dr. J. Gurdon and his colleagues showed that frog somatic cells can be reprogrammed after fusion with an enucleated oocyte, and that they can develop into a tadpole (Gurdon 1962). Reprogramming in vertebrates was also demonstrated by the creation of cloned animals from sheep (Campbell et al. 1996) and mouse (Wakayama et al. 1998) somatic cells fused with enucleated oocytes. Human and mouse ES cells can also reprogram somatic cells by cell fusion or treatment with cell extracts (Tada et al. 2001). These results indicate that somatic cells can become pluripotent following exposure to certain reprogramming factors present within oocytes and ES cells.

In 2006, Dr. S. Yamanaka and his colleagues identified these reprogramming factors (Takahashi and Yamanaka 2006). Forced expression of four transcription factors, Oct3/4, Sox2, Klf4, and cMyc, into mouse embryonic and adult fibroblasts was

able to return somatic cells to a pluripotent state. They named these cells induced pluripotent stem (iPS) cells. The first generation of iPS cells resembled ES cells in terms of morphology, proliferation, expression of some ES cell marker genes, and formation of teratomas. However, the global gene expression pattern of iPS cells differed from that of ES cells, and these iPS cells failed to produce adult chimeric mice. In 2007, germline transmission was achieved with mouse iPS cells (Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007). The current generation of iPS cells has been shown to be functionally equivalent to ES cells; they express ES cell markers, have similar gene expression profiles, form teratomas, and contribute to all cell types in chimeric animals, including the germline. Of note, recent evidence has demonstrated small but reproducible differences between ES cells and iPS cells, including differentiation potential, tumorigenic potential, gene expression profiles, epigenetic modification, expression of imprinted genes, copy number variation, proteomes and phosphoproteomes (Gore et al. 2011; Hussein et al. 2011; Kim et al. 2010; Lister et al. 2011; Phanstiel et al. 2011; Polo et al. 2010). The molecular mechanisms underlying the reprogramming process are poorly understood (Hochedlinger and Plath 2009; Yamanaka 2009).

iPS cell technology has opened up the possibility of directly reprogramming somatic cells to adopt a different cell fate. In 2010, nuclear reprogramming allowed direct conversion of somatic cells into neurons, cardiomyocytes, and blood cell progenitors without first passing through a pluripotent state (Ieda et al. 2010; Szabo et al. 2010; Vierbuchen et al. 2010). This concept, however, was not new. A pioneering study performed in 1898 by Weintraub et al. demonstrated that the expression of a single transcription factor, MyoD, is sufficient to convert fibroblasts and numerous other cell types into skeletal muscle cells (Weintraub et al. 1989). Based on these findings, it is likely that the key reprogramming factors are developmental regulators of the target cell lineage. For example, a combination of neural lineage – specific transcription factors, Ascl1, Brn2, and Myt1l, was used to convert mouse fibroblasts directly into functional neurons, known as induced neuronal (iN) cells (Vierbuchen et al. 2010). Moreover, the combination of Ascl1, Brn2, Myt1l, Lmx1a, and FoxA2 or of Ascl1, Nurr1, and Lmx1a can induce midbrain dopaminergic neurons (Caiazzo et al. 2011; Pfisterer et al. 2011). Future studies will focus on identifying the minimal set of factors sufficient for reprogramming for each cell type, particularly therapeutically significant cell types (Osakada 2011).

5.2 Neural Development

The construction of the central nervous system (CNS) is an integrated series of developmental steps, beginning with the decision of a few early embryonic cells to adopt a neural fate. Following fertilization, multiple cell divisions generate a large number of cells from the fertilized oocyte. The three germ layers, the ectoderm, endoderm, and mesoderm, arise through complex movements during gastrulation. Ectodermal cells give rise to different tissue derivatives, depending on the axial

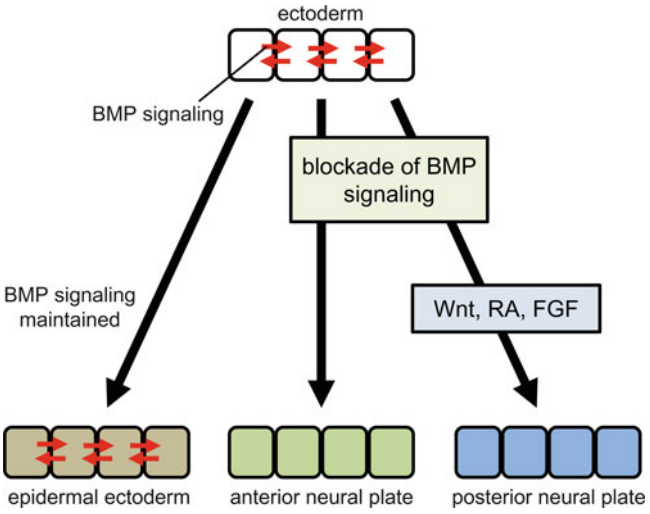


Fig. 5.3 BMP signaling and the specification of ectodermal cell fates. Ectodermal cells exposed to BMP4 differentiate into epidermal ectoderm cells. Blockade of BMP4 signaling by Chordin, Noggin, or Follistatin induces the formation of anterior neural plate tissue. Exposure of this tissue to Wnt, retinoic acid (RA), or FGF leads to the generation of posterior neural plate tissue

position. The dorsal-most ectoderm thickens to form the neural plate, which through a morphogenetic process gives rise first to the neural tube and subsequently to the CNS. Ectodermal cells at the ventral edges of the neural plate, the neural folds, come to lie at the dorsal surface of the neural tube during neurulation. Neural crest cells delaminate from this population of cells and migrate out to give rise to most of the peripheral nervous system. The ectodermal cells lying more ventral to the cranial neural plate form the placodes from which the sensory ganglia will arise. Finally, ectodermal cells on the extreme ventral side of the embryo give rise to the epidermis.

The ventral ectoderm undergoes epidermal differentiation in response to bone morphogenetic protein (BMP) ligands (Fig. 5.3). BMPs activate intracellular proteins, such as Smads, that regulate the transcription of *Gata* and *Msx* genes, which encode transcription factors. Gata and Msx proteins then inhibit *Sox* transcription to promote epidermal fate. The dorsal mesoderm, known as Spemann's organizer, expresses multiple BMP antagonists, such as Chordin, Noggin, and Follistatin, that induce neural tissue by inactivating Smad signaling (Hemmati-Brivanlou et al. 1994; Hemmati-Brivanlou and Melton 1994; Lamb et al. 1993; Sasai et al. 1994; 1995). This results in Sox protein expression, which directly activates proneural gene transcription.

After neural induction, the embryonic CNS is patterned along its anterior–posterior, dorsal–ventral, and left–right axes. The neural tube is regionalized along the antero-posterior axis, with most of the neural tube giving rise to the spinal cord and the rostral end enlarging to form the three primary brain vesicles: the prosencephalon,

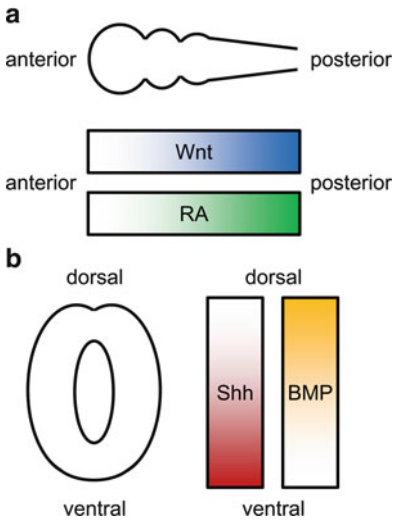


Fig. 5.4 Patterning of the nervous system. (**a**) Gradients of Wnt and retinoic acid (RA) specify the anterior–posterior axis of the neural tube. Wnt and RA posteriorize the neural tube. Suppression of Wnt and RA causes anteriorization of the neural tube. (**b**) Gradients of BMP and Shh specify the dorsal–ventral axis of the neural tube. Shh is expressed first in the notochord and later in the floorplate, and induces ventral differentiation in the neural tube. BMP is expressed in the ectoderm overlying the neural tube and then in the dorsal neural tube cells, and induces dorsal differentiation of the neural tube

mesencephalon, and rhombenchephalon. These become further subdivided into five vesicles. The prosencephalon gives rise to both the telencephalon and diencephalon. The diencephalon eventually produces the thalamus, hypothalamus, and retina (the neural retina and retinal pigmented epithelium). The mesencephalon gives rise to the midbrain, and the rhombencephalon divides into the metencephalon and myelencephalon, which form the cerebellum and medulla, respectively.

Neural induction causes the early neural plate to adopt an anterior neural fate bias. The presumptive neural plate is then patterned by caudalizing signals to generate different brain regions (Fig. 5.3). Diffusible morphogens secreted from a localized source establish concentration and activity gradients that act as a positional code to generate distinct progenitor domains, and ultimately to specify subtype identity. These signaling molecules restrict the expression of specific transcriptional factors, which go on to regulate the expression of downstream target genes that define regional identity within the nervous system.

The Wnt, retinoic acid, and FGF signaling pathways play a major role in specifying the anterior–posterior axis (Fig. 5.4a). Concomitant with anteroposterior extension and patterning of the neural plate, the dorsoventral axis is also patterned. Cell fate determination along the dorsal–ventral axis involves the action of two opposing signaling molecules: Sonic hedgehog, which originates from the notochord and later from the floor plate, and TGF-β proteins, especially BMP4, BMP7, and Activin,

which originate from the dorsal ectoderm and later from the roof plate (Fig. 5.4b). Patterning along the left–right axis also occurs during gastrulation, at the same time as anterior–posterior and dorsal–ventral axis patterning. A leading candidate for initiating asymmetry is Activin, which acts through Nodal and Lefty before any morphological differences are evident.

Stem cells are defined by their ability both to self-renew and to produce diverse cell types. During development, neural stem cells that arise from the neuroectoderm proliferate throughout the induction and patterning of the neural primordium. Stem cells in the early embryonic nervous system undergo many symmetric cell divisions to generate more stem cells, while those in the late embryo undergo asymmetric divisions to generate progenitor cells that are more restricted in their differentiation capacity (Fig. 5.1a, b). These progenitor cells eventually exit the cell cycle and differentiate into neurons, astrocytes, and oligodendrocytes (Fig. 5.1c, d).

The fates of neural stem/progenitor cells are restricted temporally, with early neural progenitors generating neurons but not glia, and later embryonic and adult neural progenitors generating both neurons and glia. However, these late neural progenitors do not produce early-born neurons, such as forebrain cholinergic neurons, midbrain dopaminergic neurons, and spinal motor neurons. In addition, neural progenitor cells maintain the regional identity of their origin; for example, it is difficult to transform telencephalon-derived neural progenitors into retinal neurons and midbrain dopaminergic neurons.

5.3 Retinal Development

The eye primordium can be identified as early as the neural plate stage. As the neural plate rolls into a tube, the lateral aspects of the anterior neural tube evaginate to form paired optic vesicles, which then fold inward to form bilayered optic cups (Fig. 5.5a–d). The inner layer of the optic cup develops into the neural retina, while the outer layer develops into the retinal pigmented epithelium (RPE) (Fig. 5.5e–i).

Eye field specification in the neural plate is regulated by a set of transcription factors, Pax6, Rx/Rax, Six3, Six6/Optx2, and Lhx2. Functional inactivation of these eye field transcription factors (EFTFs) in frogs, fish, rodents, and humans results in loss or abnormalities of the eye. Conversely, overexpression of *Pax6, Rx/Rax, Six3,* and *Six6/Optx2* expands or induces ectopic eye tissues in the vertebrate nervous system. For example, *Pax6* is expressed in the anterior neural plate at the end of gastrulation and is then restricted to the optic vesicle and lens ectoderm. Injection of *Pax6* mRNA into *Xenopus* embryos induces ectopic eyes, indicating that *Pax6* is sufficient for eye formation (Chow et al. 1999). In addition, mutations in *Pax6* result in eye malformations and reduced eye size. *Rx/Rax* is expressed in the presumptive eye field as well as the ventral diencephalon (Furukawa et al. 1997a). $Rx^{-/-}$ mice completely lack eyes, whereas overexpression of Rx in *Xenopus* and zebrafish embryos results in the formation of ectopic retinal tissue and hyperproliferation of the neural retina and the RPE (Andreazzoli et al. 1999; Chuang et al. 1999; Mathers et al. 1997). *Six3* is

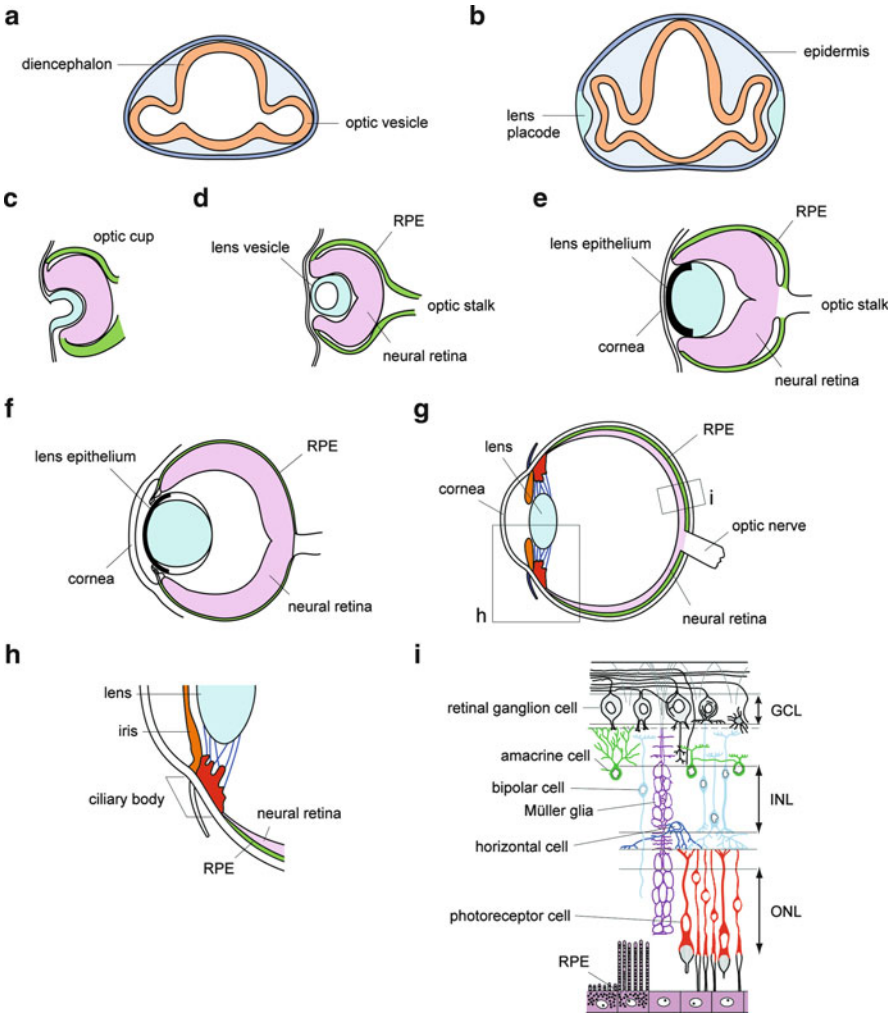


Fig. 5.5 Development of the eye. (**a**–**f**) Mouse embryos at embryonic days (E)9.5 (**a**), E10.0 (**b**), E10.5 (**c**), E11.5 (**d**), E13.5 (**e**) and E18.5 (**f**). (**g**) Adult eyes. (**h**, **i**) Magnified views of *boxed region* in panel (**g**). (**h**) Ciliary body and iris. (**i**) Cell types and layers in the adult retina. *RPE* retinal pigmented epithelium, *ONL* outer nuclear layer, *INL* inner nuclear layer, *GCL* ganglion cell layer (Figures from Experimental Medicine, 2006 by Osakada and Takahashi)

expressed in the anterior neural plate and in the presumptive eye field (Lagutin et al. 2001; Oliver et al. 1995). *Six3* plays a critical role in the formation of the forebrain, as mouse embryos lacking Six3 function lack most of the head structures anterior to the midbrain (Lagutin et al. 2003). The specific role of *Six3* in eye development, however, remains unknown due to the early head truncation phenotype of *Six3* mutants. In addition, *Six6/Optx2* plays a role in proliferation of retinal progenitors. These EFTFs interact directly with one another and form a self-regulating feedback network, though it remains unclear how this coordinated expression is established.

Table 5.1 Intrinsic factors regulating retinal cell differentiation

Cell type	Homeobox genes	bHLH genes
Photoreceptor cells	Crx/Otx2	NeuroD/Mash1
Horizontal cells	Pax6/Six3/Prox1	Math3
Bipolar cells	Chx10	Mash1/Math3
Amacrine cells	Pax6/Six3	NeuroD/Math3
Ganglion cells	Pax6	Math5
Müller glia	Rx	Hes1/Hes5

Little is known about the extracellular signaling molecules that regulate the EFTFs. Wnt1 and Wnt8b activate a Wnt/ß-catenin pathway, and cause reduction of the eye field by suppressing Rx and Six3 expression when overexpressed in *Xenopus* embryos. Wnt11 activates the non-canonical Wnt pathway and causes enlarged eyes when overexpressed in *Xenopus* (Cavodeassi et al. 2005). Overexpression of Frizzled-3, a Wnt receptor, also results in formation of multiple ectopic eyes in *Xenopus.* In mutants lacking the function of Dickkopf-1 (Dkk-1), an inhibitor of canonical Wnt signaling, cranial structures anterior to the midbrain are lost, including the eye (Mukhopadhyay et al. 2001). In *Xenopus*, the BMP inhibitor Noggin induces the expression of EFTFs, including Pax6, Rx, Six3, Six6, and Lhx2 (Zuber et al. 2003). In addition, overexpression of ectonucleoside triphosphate diphosphohydrolase 2 (E-NTPDase2), an ectoenzyme that converts ATP to ADP, causes ectopic eye-like structures in *Xenopus*, while downregulation of endogenous *E-NTPDase2* decreases *Rx1* and *Pax6* expression (Masse et al. 2007). Alterations to the *E-NTPDase2* locus on human chromosome 9 cause severe head and eye defects. Finally, Notch signaling in retinal specification has been also reported. Overexpression of a constitutively active Notch internal cytoplasmic domain (NICD) induces expression of *Pax6* and the formation of ectopic eyes in *Xenopus* (Onuma et al. 2002). Hes1, a component of the Notch signaling pathway, is expressed in the anterior neural plate and subsequently in the optic cup (Lee et al. 2005). Loss of *Hes1* alone results in reduced eye size, while combined loss of *Hes1* and *Pax6* or *Hes1* and *Hes5* prevents optic cup formation (Hatakeyama et al. 2004; Lee et al. 2005).

During retinal development, stem cells proliferate extensively to increase their cell number and give rise to distinct subtypes of cells over time by changing their competency. The seven cell types in the retina are born from retinal stem cells in the following temporal sequence: retinal ganglion cells, cone photoreceptors, amacrine cells, and horizontal cells, followed by rod photoreceptors, bipolar cells, and Müller glia. These cells are organized into three cell layers: rod and cone photoreceptors in the outer nuclear layer (ONL), Müller glia, horizontal, bipolar, and amacrine cells in the inner nuclear layer (INL), and ganglion and displaced amacrine cells in the ganglion cell layer (GCL) (Fig. 5.5i).

The differentiation of each retinal cell type is a highly complex process requiring both extrinsic and intrinsic factors. Intrinsic factors include combinations of bHLH and homeodomain transcription factors (Table 5.1) that work together to specify retinal cell subtype. It is likely that homeodomain factors regulate layer specificity but not neuronal fate, while bHLH proteins determine neuronal fate within the homedomain factor–specified layers. For example, the generation of

Table 5.2 Extrinsic factors regulating retinal cell differentiation

Cell type	Soluble factor
Photoreceptor cells	(+) Retinoic acid
	(+) Taurine,
	(+) Thyroid hormone
	(+) Shh
	(+) FGF
	(−) CNTF
Horizontal cells	
Bipolar cells	(+) CNTF
Amacrine cells	
Ganglion cells	(−) Shh
Müller glia	(−) Retinoic acid
	(−) FGF

(+) promotes differentiation, (−) inhibits differentiation

photoreceptors is regulated by Crx, Otx2 (homeobox gene products), and NeuroD (basic helix-loop-helix proteins). Mice lacking *Crx* function exhibit deficits in outer segment formation in their photoreceptors (Chen et al. 1997; Furukawa et al. 1997b). On the other hand, overexpression of Crx in P0 progenitors promotes the photoreceptor formation and inhibits amacrine fate *in vivo*. Conditional *Otx2* knockout mice lack photoreceptor differentiation (Nishida et al. 2003), while loss of NeuroD, which is expressed in photoreceptors and amacrine cells (Morrow et al. 1999), results in moderately decreased photoreceptor number.

Extrinsic factors regulating retinal differentiation have also been identified (Table 5.2). For example, retinoic acid promotes photoreceptor differentiation, and inhibition of endogenous retinoic acid synthesis results in a reduction in rod differentiation (Hyatt et al. 1996). The amino acid taurine promotes rod differentiation via the α2 glycine receptor and the $GABA_A$ receptor (Altshuler and Cepko 1992; Young and Cepko 2004).

Once cells are committed to a particular fate, they migrate to stereotyped positions throughout the laminated retina and establish synaptic connections to other neurons. Synapse formation proceeds centrifugally from the inner to the outer retina, first among the horizontal connections within the plexiform layers, followed by vertical connections between layers.

5.4 Adult Neurogenesis

For many decades, it was believed that neurons in the adult mammalian CNS could not regenerate after injury, as postulated by Ramón y Cajal in 1913. However, recent evidence has overturned this long-held dogma. Neural stem cells are present not only during embryonic development, but also in the adult brains of mammals, including humans (Eriksson et al. 1998; Reynolds et al. 1992; Reynolds and Weiss

1992; Sanai et al. 2004). The production of neurons occurs primarily during nervous system development, but throughout adulthood, new neurons are generated in two locations of the brain under normal conditions: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus.

Adult neural stem cells can self-renew and are multipotent, differentiating into three types of neural cells: neurons, astrocytes, and oligodendrocytes. Neurons born in the adult SVZ migrate over a great distance through the rostral migratory stream and become granule neurons and periglomerular neurons in the olfactory bulb (Lois and Alvarez-Buylla 1994; Lois et al. 1996). Neurons born in the adult SGZ migrate into the granule cell layer of the dentate gyrus and become dentate granule cells. These newborn neurons in the adult brain integrate into the existing circuitry, and are able to receive and send functional signals similar to neurons born during embryogenesis. Increasing evidence suggests that adult neural stem cells significantly contribute to specialized neural functions under physiological and pathological conditions, such as learning, memory, olfaction, depression, epilepsy, and stroke. Adult neurogenesis can be divided into three major steps: proliferation, neuronal determination, and maturation. These different developmental stages are regulated by distinct processes.

Though the existence of neural stem cells in the adult brain has been established, their precise identity remains controversial, because the SVZ and SGZ are heterogeneous in terms of cell morphology and marker expression (Chojnacki et al. 2009). In the adult SVZ, neural stem cells correspond to SVZ astrocytes (type B cells), which are derived from radial glia, the neural stem cells of the embryonic and early postnatal brain (Doetsch et al. 1997, 1999). Type B cells generate transit amplifying cells (type C cells) that give rise to young neurons or neuroblasts (type A cells). Type B cells express GFAP, and are quiescent and less susceptible to anti-mitotic treatment (Doetsch et al. 1999), while type C cells are most frequently labeled with BrdU. Type A cells express PSA-NCAM and doublecortin, both of which are associated with neuronal migration. On the other hand, in the adult SGZ, radial glia-like cells, whose cell bodies are located in the SGZ and whose long processes extend through the granule cell layer into the inner molecular layer, are neural stem cells (type 1 cells) (Fig. 5.6). Type 1 cells are infrequently labeled by BrdU and generate type 2 cells, which possess short processes and high proliferative activity. Type 1 cells express GFAP, while type 2 cells express Nestin but not GFAP.

Neuroblasts originating from SVZ progenitors migrate tangentially toward the olfactory bulb along the rostral migratory stream (Lois and Alvarez-Buylla 1994; Lois et al. 1996). Once they arrive in the olfactory bulb, migrating neuroblasts detach from the chain and migrate radially into the granule and glomerular cell layers of the olfactory bulb. Newborn neurons go through morphological and physiological development, and integrate as granule neurons in the granule cell layer and as periglomerular neurons in the glomerular layer. Interestingly, recent evidence has demonstrated that SVZ type B cells are heterogeneous and predetermined to generate specific types of neurons in the olfactory bulb (Merkle et al. 2007). SVZ type B cells in different locations within the germinal region generate different types of interneurons.

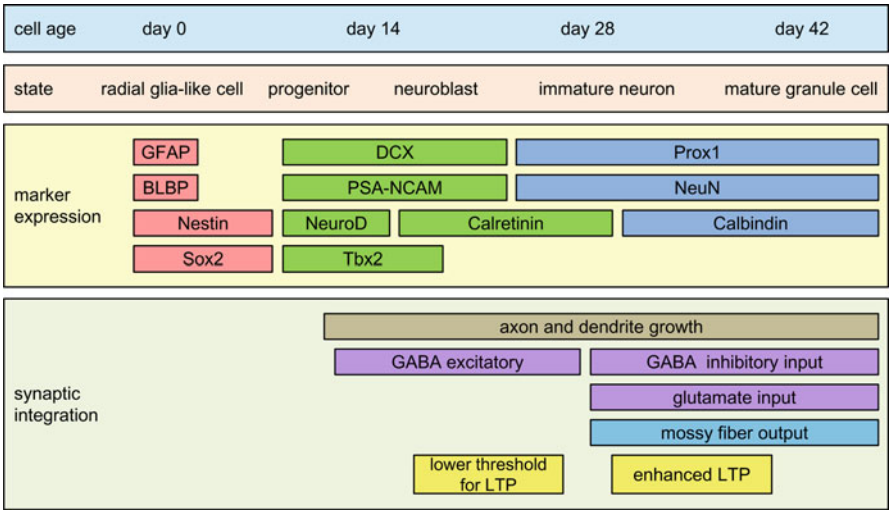


Fig. 5.6 Developmental stages in adult hippocampal neurogenesis. Development of newly generated neurons in the dentate gyrus proceeds through a series of stages characterized by expression of specific markers, morphogenesis, synapse formation, acquisition of electrophysiological properties, and functional integration into neural circuits. *LTP* long-term potentiation

During the maturation process in adult neurogenesis, the first functional synaptic innervation of progenitor cells by hippocampal circuitry is GABAergic (Fig. 5.6). Similar to immature neurons in the developing brain, newborn granule cells initially become depolarized in response to GABA because of their higher intracellular concentration of chloride ions (Ben-Ari 2002; Ge et al. 2006). The response to GABA switches from depolarization to hyperpolarization at 2–4 weeks after neuronal birth, which coincides with the growth of dendritic spines and the onset of glutamatergic responses. Within this time window, new neurons have lower thresholds for long-term potentiation (Ge et al. 2007; Schmidt-Hieber et al. 2004). Newborn neurons in the dentate gyrus display typical features of mature granule cells at 4 weeks of age, but they continue to change both physiologically and morphologically. They have round cell bodies in the GCL, complex spiny dendrites reaching the hippocampal fissure, and an axon that projects through the hilus toward CA3. The amplitude of long-term potentiation is greater in new neurons 4–6 weeks after birth. This may be mediated by the NR2B subunit of the NMDA receptor. Once they mature, newborn granule cells receive glutamatergic (excitatory) and GABAergic (inhibitory) inputs, send functional synaptic projections to CA3 pyramidal cells and hilar interneurons by releasing the neurotransmitter glutamate, and become completely integrated into the hippocampal circuitry in the dentate gyrus.

Adult neurogenesis contributes to both the plasticity and regenerative capacity of the adult brain, and opens the possibility for potential future therapeutic applications based on the manipulation of this regenerative capacity. In particular, a better understanding of the basic mechanisms regulating adult neurogenesis may provide

the foundation for treating neurodegenerative diseases, since adult neurogenesis in both the SVZ and SGZ declines during aging. Many lines of evidence indicate that neurotransmitters (GABA, glutamate, dopamine, acetylcholine and serotonin) (Cameron et al. 1995; Hoglinger et al. 2004; Liu et al. 2005), hormones (corticosteroids and prolactin), growth factors (FGF, EGF, BDNF, CNTF, IGF, VEGF, Shh, and Wnt) (Lai et al. 2003; Lie et al. 2005), and physiological and pathological stimuli (environmental enrichment, electroconvulsive shock stimulation, stress and seizures) (Kempermann et al. 1997; Mirescu and Gould 2006; van Praag et al. 1999; Warner-Schmidt and Duman 2006) affect adult neurogenesis.

5.5 Retinal Regeneration

Regeneration in the CNS necessitates the reacquisition of pre-existing neural structures and function following injury and disease. The strategies for regeneration can be classified into two approaches: (i) activation of endogenous neural stem cells and (ii) transplantation of lost cell types (Goldman 2005; Osakada et al. 2010; Osakada and Takahashi 2009).

Visual impairment is usually caused by specific loss of different cell populations within the retina (Osakada et al. 2010; Osakada and Takahashi 2009). For example, glaucoma is a retinal degenerative disease in which the retinal ganglion cells (RGCs) forming the optic nerve are selectively lost. In retinitis pigmentosa, photoreceptors are lost due to genetic mutation (Hartong et al. 2006; Wright et al. 2010). In age-related macular degeneration (AMD), degeneration of the retinal pigmented epithelium (RPE) is followed by loss of photoreceptors (Rattner and Nathans 2006). Since first order neurons are selectively affected in retinitis pigmentosa and AMD, the neural circuitry mediating higher order visual processing is maintained in the early phase of degeneration (Bi et al. 2006; Busskamp et al. 2010; Humayun et al. 2003; Lagali et al. 2008; Mazzoni et al. 2008). Thus, repair of photoreceptor or RPE cells may permit recovery of visual function. It should be noted that retinal regeneration differs from regeneration of the optic nerve. Retinal regeneration aims to replace photoreceptors and reconstruct their synapses with proximal secondary neurons (bipolar cells and horizontal cells) within the retina. In contrast, optic nerve regeneration to treat glaucoma and other diseases requires replacement of RGCs and reconstruction of distant synaptic connections to the brain.

The capacity for adult neurogenesis in the retina is greatest in fish and amphibians. The ciliary margin zone (CMZ) of fish and amphibians contributes to retinal growth throughout the animal's life. In response to damage, retinal progenitors in the CMZ generate new retinal neurons in amphibians, fish, and birds. Thus, the CMZ resembles other regions containing neural stem cells, like the SVZ and the SGZ. Interestingly, sphere culture methods have shown that retinal stem cells persist in the mammalian ciliary epithelium (Ahmad et al. 2000; Tropepe et al. 2000). Moreover, iris cells from birds and mammals can generate retinal neurons *in vitro* (Haruta et al. 2001; Sun 2006). In amphibians, the RPE is the primary source of new

retinal progenitors (Reh et al. 1987). After removal of the retina, the RPE loses pigmentation and proliferates to generate two new epithelial layers, a pigmented layer and a non-pigmented layer. The non-pigmented layer begins to express genes typical of retinal progenitors and undergoes extensive cell division to produce neurons for the new retina (Reh and Nagy 1987). In fish, birds, and mammals, Müller glia act as endogenous progenitors and generate new neurons in response to damage (Fischer and Reh 2001; Ooto et al. 2004).

In adult mammals, Müller glia have the potential to generate retinal neurons after injury *in vivo* (Karl et al. 2008; Ooto et al. 2004). The neural stem cell properties of Müller glia have been also verified *in vitro*. Dissociated Müller glia derived from injured retinas form neurospheres *in vitro*, which can differentiate into neurons and glia (Das et al. 2006). In addition, Müller glia–derived progenitors can be identified and purified as a side population of cells by the Hoechst dye efflux, another characteristic of progenitor cells (Das et al. 2006). After transplantation into the retina, these Müller glia – derived neurosphere cells can differentiate into retinal neurons.

Several lines of evidence support a close relationship between Müller glia and retinal progenitors. Recent gene expression profiling studies have demonstrated a large degree of overlap in the genes expressed in the Müller glia and late retinal progenitors. Moreover, the proliferation and differentiation of Müller glia–derived progenitors can be regulated by both intrinsic (homeobox and basic helix-loop-helix genes) and extrinsic (Wnt, Notch, Shh, FGF, EGF and BDNF) factors, similar to what has been observed in retinal progenitors during eye development (Das et al. 2006; Harada et al. 2011; Osakada et al. 2007; Wan et al. 2007). However, how Müller glia in the mammal reacquire neurogenic potential is still unknown. Several lines of evidence have demonstrated that activation of Shh, Wnt and Notch is sufficient to stimulate Müller glia to enter a neurogenic mode in the absence of injury (Del Debbio et al. 2010; Wan et al. 2007). Epigenetic modifications in Müller glia might be also involved in reacquisition of neurogenic potential. Since Müller glia are a potential source of regenerating cells in the adult mammalian retina, developing drugs that target these cells is a promising approach that may lead to new retinal regeneration therapies (Osakada and Takahashi 2009).

For photoreceptor transplantation, cells from the developing retina can be used as a donor source for transplantation. Importantly, integration of donor rod photoreceptors in the host retina requires rod photoreceptors corresponding to postnatal days 3–6 (MacLaren et al. 2006). However, use of human fetal tissue presents ethical problems, and the quantity of available fetal retinal cells is limited. Thus, *in vitro* expansion of retinal cells derived from stem/progenitor cells, if possible, would be ideal. When adult stem cells from the SGZ are transplanted into the developing eye, they integrate into the retina and exhibit morphologies and positions characteristic of Müller, amacrine, bipolar, horizontal, and photoreceptor cells (Takahashi et al. 1998). However, none acquire end-stage markers unique to retinal neurons. Thus, adult brain–derived stem cells cannot adopt retinal fates even when exposed to the cues present during retinal development. Although the brain and the retina are both generated from the ectodermally-derived neural tube, neural progenitors in different CNS regions differ in their competence to generate specific types of mature neurons.

Alternatively, retinal progenitors in the embryonic retina can be expanded *in vitro* and can differentiate into various types of retinal neurons; however, they lose their ability to differentiate into photoreceptors following massive expansion (Akagi et al. 2003).

The somatic progenitors in adult eye tissue are another potential source of donor cells. The ciliary marginal zone has been reported to contain stem cells even in adults (Ahmad et al. 2000; Tropepe et al. 2000). When cultured *in vitro*, these cells give rise to retinal neurons, including photoreceptors. Iris-derived cells have also been reported to generate retinal neurons (Haruta et al. 2001). Adult tissues offer the advantage that they can be used as autografts, which do not cause immune rejection. Autologous iris tissue can be feasibly obtained by peripheral iridectomy. Unlike the hippocampus, both the ciliary margin and the iris derive from the optic vesicle and optic cup, suggesting that they may be more competent than brain stem cells to generate retinal neurons. However, cells differentiated from adult somatic progenitors in the eye express several photoreceptor marker proteins, but not all the genes responsible for photoreceptor function. Thus, it is likely that the generation of functional photoreceptors requires a recapitulation of the normal process of retinal development.

ES cells are another potential source of donor cells for retinal transplantation. Based on our knowledge of embryonic development, we have developed methods of inducing stepwise differentiation of ES cells into retinal progenitors (Rx^+, $Mitf^+$, $Pax6^+$, $Chx10^+$), photoreceptors (Crx^+, Nrl^+, $rhodopsin^+$, $recoverin^+$) and RPE ($Mitf^+$, $ZO1^+$, $RPE65^+$). (Ikeda et al. 2005; Osakada et al. 2008, 2009a, b) (Fig. 5.7a, b). Surprisingly, optic cup structure can be induced from three-dimension culture of mouse ES cells, indicating some self-organizing capacity that might be harnessed (Eiraku et al. 2011). Transplantation of ES cell–derived photoreceptor sheets might also be an effective approach (Aramant and Seiler 2004). While somatic progenitors derived from the ciliary body or iris are limited in both differentiation potential and proliferation capacity, human ES cells can generate a large number of retinal cells. Indeed, transplantation of photoreceptors or RPE derived from human ES cells has been reported to restore some visual function (Lamba et al. 2009). Regeneration of the RPE is also important because it is essential for photoreceptor function; indeed, RPE degeneration causes secondary photoreceptor degeneration. Several promising lines of evidence indicate that transplantation of ES cell–derived RPE can prevent photoreceptor degeneration in an RPE degeneration model, RCS rats (Haruta et al. 2004; Idelson et al. 2009; Lund et al. 2006).

iPS cell technology provided a paradigm shift not only in our understanding of cell biology, but also in regenerative medicine approaches (Takahashi et al. 2007; Takahashi and Yamanaka 2006; Yu et al. 2007). iPS cells are functionally equivalent to ES cells and therefore share the same advantages as ES cells: pluripotency and proliferation capacity. However, for clinical applications, they offer the additional benefits of avoiding problems faced by human ES cell technology: the ethical problems surrounding the use of human embryos and the biological problem of tissue rejection (Takahashi et al. 2007; Yu et al. 2007). Thus, patient-specific, customized cell therapy might be possible. The retinal differentiation methods for ES cells are

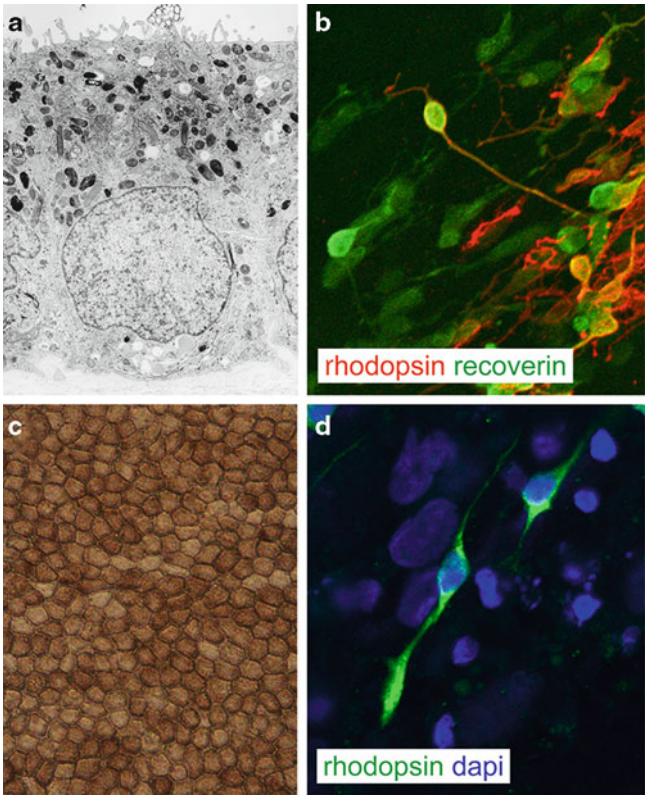


Fig. 5.7 Differentiation of retinal cells from human ES and iPS cells. (**a**) Electron micrograph of human ES cell–derived RPE cells. (**b**) Human ES cell–derived rod photoreceptor cells express both rhodopsin and recoverin. (**c**, **d**) Generation of RPE (**c**) and photoreceptors (**d**) from human iPS cells (Figures from Nature Biotechnology, 2008 by Osakada et al.)

applicable to iPS cells (Hirami et al. 2009; Osakada et al. 2009b) (Fig. 5.7c, d). Transplantation of human iPS cell–derived RPE can rescue photoreceptors in an animal model of RPE degeneration and has a therapeutic potential (Carr et al. 2009). By contrast, FACS-sorted photoreceptor cells from human iPS cells cannot integrate into the normal mouse retina and are inefficient for functional restoration, although unsorted iPS cell–derived cells can be transplanted and survive in the retina (Lamba et al. 2010).

Nuclear reprogramming of somatic cells directly to retinal neurons and RPE could be also a promising approach to obtain retinal cells more quickly and more safely (Osakada 2011). One key issue in autologous transplantation for genetic disorders is that genetic defects due to mutations or deletion need to be repaired before transplantation. Gene correction by homologous recombination or zinc finger nuclease technology is feasible in mouse iPS cells and human iPS cells (Hanna et al. 2007; Liu et al. 2011; Yusa et al. 2011). For successful retinal regeneration, methods of

purifying donor retinal cells and optimizing host conditions, as well as use of animal models of human diseases to determine the efficacy and safety of treatments, will be crucial.

5.6 Conclusions and Perspectives

Over the past decade, significant progress has been made in stem cell biology. A better understanding of stem cells has shed light on the processes involved in embryonic development, adult neurogenesis, and regeneration. In particular, the discoveries of adult neural stem cells, ES cells, and iPS cells will stimulate both basic research and applied biomedical study.

It has been established that neurogenesis and neural regeneration take place even in the mammalian adult CNS, but many questions must still be resolved. For example, what are the physiological roles of neural stem cells in the adult brain? Why do only two regions generate new neurons in the intact adult brain? What is the difference between neurogenic and non-neurogenic regions? Can neurogenesis or neural regeneration be induced in non-neurogenic regions? How are new neurons integrated into preexisting neural circuits? How did the difference in regeneration capacity among species arise through evolution? A detailed understanding of stem/progenitor cells in the adult CNS will be important for therapeutic applications for CNS repair.

Despite tremendous progress in stem cell biology, there is still a large gap between the cellular and behavioral approaches towards understanding the pathophysiological roles of new neurons in the adult CNS (Aimone et al. 2010; Lledo et al. 2006). A straightforward way to study their functional contribution is to eliminate new neurons in the adult CNS by irradiation or administration of antimitotic drugs such as methylazoxymethanol acetate and temozolomide, which kill dividing cells in the adult brain (Madsen et al. 2003; Shors et al. 2001). However, these approaches cannot uncover the properties of newly generated neuronal connections. Methods to analyze the integration of newly generated neurons or transplanted cells into existing neural networks are not well established at present. Addressing these questions at the circuit level will require visualizing newly formed connections, monitoring and manipulating the activity of these connections, and assessing the behavioral outcome. Recombinant viral vectors will be powerful tools for these purposes (Luo et al. 2008; Osakada et al. 2011).

During vertebrate embryogenesis, the nervous system primordium arises from uncommitted ectoderm during gastrulation. While much is known about the mechanism of neural induction in amphibians, comparatively little is known about this process in mammals, in part because good experimental systems for *in vitro* neural differentiation comparable to the animal cap assay commonly used in *Xenopus* studies are still lacking in mice. Unlike the amphibian animal cap, which is large and easy to prepare in large quantities, the mammalian ICM and epiblast are tiny and technically demanding to handle. However, *in vitro* differentiation of ES cells

recapitulates many aspects of embryonic development *in vivo* (Hansen et al. 2011; Osakada and Takahashi 2011). Indeed, the spatial and temporal aspects of neurogenesis can be recapitulated and manipulated in response to morphogens in ES cell culture (Gaspard et al. 2008; Mizuseki et al. 2003; Watanabe et al. 2005; Wichterle et al. 2002). Intriguingly, ES cells can generate the self-organized laminar structure of the cortex, including four distinct zones (ventricular, early and late cortical-plate, and Cajal–Retzius cell zones) along the apico–basal axis (Eiraku et al. 2008), and the optic cup structure in three dimension culture of ES cells (Eiraku et al. 2011). Differentiation culture of ES cells and iPS cells provides a versatile and powerful *in vitro* tool complementary to *in vivo* approaches. Such studies will provide an improved understanding of the mechanisms of mammalian development.

In addition to providing a promising approach towards cell transplantation therapy to treat disease or injury, stem cell technology has the potential to revolutionize drug discovery, making models available for primary screening, toxicity evaluation, and metabolic profiling. Mouse ES cells are already in use in drug discovery, and high-throughput screening is currently being developed. Since human models for disease are highly desirable, human ES cells and iPS cells will be powerful tools for drug discovery. In particular, the generation of patient-specific or disease-specific human iPS cells will be a strong tool for studying disease mechanisms, screening drugs, and developing new therapies (Brennand et al. 2011; Jin et al. 2011). For genetic diseases, iPS cells provide a new opportunity to analyze the molecular pathways that lead to disease pathogenesis at the cellular level (Jin et al. 2011). Moreover, drug effects during clinical treatment might be predicted and analyzed using iPS cells from patients, permitting personalized optimization of drug treatment.

Acknowledgements We thank Yoshiki Sasai (RIKEN), Akinori Akaike (Kyoto University), Edward M. Callaway (The Salk Institute for Biological Studies), and E. J. Chichilnisky (The Salk Institute for Biological Studies) for their continued support and challenging discussions, and members of the Takahashi laboratory, the Sasai laboratory, the Akaike laboratory, the Callaway laboratory, and the Chichilnisky laboratory for stimulating discussions. We are grateful for support from the Leading Project for Realization of Regenerative Medicine (M.T.), Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (F.O.), the Mochida Memorial Foundation for Medical and Pharmaceutical Research (F.O.), the Kanae Foundation for the Promotion of Medical Science (F.O.), the Uehara Memorial Foundation, and the Naito Foundation (F.O.). We apologize to those authors whose articles, although relevant to this subject, have not been cited in this review due to space limitations.

References

Ahmad I, Tang L, Pham H (2000) Identification of neural progenitors in the adult mammalian eye. Biochem Biophys Res Commun 270:517–521

Aimone JB, Deng W, Gage FH (2010) Adult neurogenesis: integrating theories and separating functions. Trends Cogn Sci 14:325–337

Akagi T, Haruta M, Akita J, Nishida A, Honda Y, Takahashi M (2003) Different characteristics of rat retinal progenitor cells from different culture periods. Neurosci Lett 341:213–216

Altshuler D, Cepko C (1992) A temporally regulated, diffusible activity is required for rod photoreceptor development in vitro. Development 114:947–957

Andreazzoli M, Gestri G, Angeloni D, Menna E, Barsacchi G (1999) Role of Xrx1 in Xenopus eye and anterior brain development. Development 126:2451–2460

Aramant RB, Seiler MJ (2004) Progress in retinal sheet transplantation. Prog Retin Eye Res 23:475–494

Ben-Ari Y (2002) Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci 3:728–739

Bi A, Cui J, Ma YP, Olshevskaya E, Pu M, Dizhoor AM, Pan ZH (2006) Ectopic expression of a microbial-type rhodopsin restores visual responses in mice with photoreceptor degeneration. Neuron 50:23–33

Brennand KJ, Simone A, Jou J, Gelboin-Burkhart C, Tran N, Sangar S, Li Y, Mu Y, Chen G, Yu D et al (2011) Modelling schizophrenia using human induced pluripotent stem cells. Nature 473:221–225

Busskamp V, Duebel J, Balya D, Fradot M, Viney TJ, Siegert S, Groner AC, Cabuy E, Forster V, Seeliger M et al (2010) Genetic reactivation of cone photoreceptors restores visual responses in retinitis pigmentosa. Science 329:413–417

Caiazzo M, Dell'Anno MT, Dvoretskova E, Lazarevic D, Taverna S, Leo D, Sotnikova TD, Menegon A, Roncaglia P, Colciago G et al (2011) Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. Nature 476:224–227

Cameron HA, McEwen BS, Gould E (1995) Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. J Neurosci 15:4687–4692

Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996) Sheep cloned by nuclear transfer from a cultured cell line. Nature 380:64–66

Carr AJ, Vugler AA, Hikita ST, Lawrence JM, Gias C, Chen LL, Buchholz DE, Ahmado A, Semo M, Smart MJ et al (2009) Protective effects of human iPS-derived retinal pigment epithelium cell transplantation in the retinal dystrophic rat. PLoS One 4:e8152

Cavodeassi F, Carreira-Barbosa F, Young RM, Concha ML, Allende ML, Houart C, Tada M, Wilson SW (2005) Early stages of zebrafish eye formation require the coordinated activity of Wnt11, Fz5, and the Wnt/beta-catenin pathway. Neuron 47:43–56

Chen S, Wang QL, Nie Z, Sun H, Lennon G, Copeland NG, Gilbert DJ, Jenkins NA, Zack DJ (1997) Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron 19:1017–1030

Chojnacki AK, Mak GK, Weiss S (2009) Identity crisis for adult periventricular neural stem cells: subventricular zone astrocytes, ependymal cells or both? Nat Rev Neurosci 10:153–163

Chow RL, Altmann CR, Lang RA, Hemmati-Brivanlou A (1999) Pax6 induces ectopic eyes in a vertebrate. Development 126:4213–4222

Chuang JC, Mathers PH, Raymond PA (1999) Expression of three Rx homeobox genes in embryonic and adult zebrafish. Mech Dev 84:195–198

Das AV, Mallya KB, Zhao X, Ahmad F, Bhattacharya S, Thoreson WB, Hegde GV, Ahmad I (2006) Neural stem cell properties of Muller glia in the mammalian retina: regulation by Notch and Wnt signaling. Dev Biol 299:283–302

Del Debbio CB, Balasubramanian S, Parameswaran S, Chaudhuri A, Qiu F, Ahmad I (2010) Notch and Wnt signaling mediated rod photoreceptor regeneration by Muller cells in adult mammalian retina. PLoS One 5:e12425

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A (1997) Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J Neurosci 17:5046–5061

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716

Eiraku M, Watanabe K, Matsuo-Takasaki M, Kawada M, Yonemura S, Matsumura M, Wataya T, Nishiyama A, Muguruma K, Sasai Y (2008) Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. Cell Stem Cell 3:519–532

Eiraku M, Takata N, Ishibashi H, Kawada M, Sakakura E, Okuda S, Sekiguchi K, Adachi T, Sasai Y (2011) Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 472:51–56

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313–1317

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156
Fischer AJ, Reh TA (2001) Muller glia are a potential source of neural regeneration in the postnatal chicken retina. Nat Neurosci 4:247–252
Furukawa T, Kozak CA, Cepko CL (1997a) rax, a novel paired-type homeobox gene, shows expression in the anterior neural fold and developing retina. Proc Natl Acad Sci U S A 94:3088–3093
Furukawa T, Morrow EM, Cepko CL (1997b) Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell 91:531–541
Gaspard N, Bouschet T, Hourez R, Dimidschstein J, Naeije G, van den Ameele J, Espuny-Camacho I, Herpoel A, Passante L, Schiffmann SN et al (2008) An intrinsic mechanism of corticogenesis from embryonic stem cells. Nature 455:351–357
Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H (2006) GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 439:589–593
Ge S, Yang CH, Hsu KS, Ming GL, Song H (2007) A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. Neuron 54:559–566
Goldman S (2005) Stem and progenitor cell-based therapy of the human central nervous system. Nat Biotechnol 23:862–871
Gore A, Li Z, Fung HL, Young JE, Agarwal S, Antosiewicz-Bourget J, Canto I, Giorgetti A, Israel MA, Kiskinis E et al (2011) Somatic coding mutations in human induced pluripotent stem cells. Nature 471:63–67
Gotz M, Huttner WB (2005) The cell biology of neurogenesis. Nat Rev Mol Cell Biol 6:777–788
Gurdon JB (1962) The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J Embryol Exp Morphol 10:622–640
Hanna J, Wernig M, Markoulaki S, Sun CW, Meissner A, Cassady JP, Beard C, Brambrink T, Wu LC, Townes TM, Jaenisch R (2007) Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science 318:1920–1923
Hansen DV, Rubenstein JL, Kriegstein AR (2011) Deriving excitatory neurons of the neocortex from pluripotent stem cells. Neuron 70:645–660
Harada C, Guo X, Namekata K, Kimura A, Nakamura K, Tanaka K, Parada LF, Harada T (2011) Glia- and neuron-specific functions of TrkB signalling during retinal degeneration and regeneration. Nat Commun 2:189
Hartong DT, Berson EL, Dryja TP (2006) Retinitis pigmentosa. Lancet 368:1795–1809
Haruta M, Kosaka M, Kanegae Y, Saito I, Inoue T, Kageyama R, Nishida A, Honda Y, Takahashi M (2001) Induction of photoreceptor-specific phenotypes in adult mammalian iris tissue. Nat Neurosci 4:1163–1164
Haruta M, Sasai Y, Kawasaki H, Amemiya K, Ooto S, Kitada M, Suemori H, Nakatsuji N, Ide C, Honda Y, Takahashi M (2004) In vitro and in vivo characterization of pigment epithelial cells differentiated from primate embryonic stem cells. Invest Ophthalmol Vis Sci 45:1020–1025
Hatakeyama J, Bessho Y, Katoh K, Ookawara S, Fujioka M, Guillemot F, Kageyama R (2004) Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development 131:5539–5550
Hemmati-Brivanlou A, Melton DA (1994) Inhibition of activin receptor signaling promotes neuralization in Xenopus. Cell 77:273–281
Hemmati-Brivanlou A, Kelly OG, Melton DA (1994) Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. Cell 77:283–295
Hirami Y, Osakada F, Takahashi K, Okita K, Yamanaka S, Ikeda H, Yoshimura N, Takahashi M (2009) Generation of retinal cells from mouse and human induced pluripotent stem cells. Neurosci Lett 458:126–131
Hochedlinger K, Plath K (2009) Epigenetic reprogramming and induced pluripotency. Development 136:509–523
Hoglinger GU, Rizk P, Muriel MP, Duyckaerts C, Oertel WH, Caille I, Hirsch EC (2004) Dopamine depletion impairs precursor cell proliferation in Parkinson disease. Nat Neurosci 7:726–735
Humayun MS, Weiland JD, Fujii GY, Greenberg R, Williamson R, Little J, Mech B, Cimmarusti V, Van Boemel G, Dagnelie G, de Juan E (2003) Visual perception in a blind subject with a chronic microelectronic retinal prosthesis. Vision Res 43:2573–2581

Hussein SM, Batada NN, Vuoristo S, Ching RW, Autio R, Narva E, Ng S, Sourour M, Hamalainen R, Olsson C et al (2011) Copy number variation and selection during reprogramming to pluripotency. Nature 471:58–62

Hyatt GA, Schmitt EA, Fadool JM, Dowling JE (1996) Retinoic acid alters photoreceptor development in vivo. Proc Natl Acad Sci U S A 93:13298–13303

Idelson M, Alper R, Obolensky A, Ben-Shushan E, Hemo I, Yachimovich-Cohen N, Khaner H, Smith Y, Wiser O, Gropp M et al (2009) Directed differentiation of human embryonic stem cells into functional retinal pigment epithelium cells. Cell Stem Cell 5:396–408

Ieda M, Fu JD, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG, Srivastava D (2010) Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell 142:375–386

Ikeda H, Osakada F, Watanabe K, Mizuseki K, Haraguchi T, Miyoshi H, Kamiya D, Honda Y, Sasai N, Yoshimura N et al (2005) Generation of Rx+/Pax6+ neural retinal precursors from embryonic stem cells. Proc Natl Acad Sci U S A 102:11331–11336

Jin ZB, Okamoto S, Osakada F, Homma K, Assawachananont J, Hirami Y, Iwata T, Takahashi M (2011) Modeling retinal degeneration using patient-specific induced pluripotent stem cells. PLoS One 6:e17084

Karl MO, Hayes S, Nelson BR, Tan K, Buckingham B, Reh TA (2008) Stimulation of neural regeneration in the mouse retina. Proc Natl Acad Sci U S A 105:19508–19513

Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493–495

Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, Kim J, Aryee MJ, Ji H, Ehrlich LI et al (2010) Epigenetic memory in induced pluripotent stem cells. Nature 467:285–290

Lagali PS, Balya D, Awatramani GB, Munch TA, Kim DS, Busskamp V, Cepko CL, Roska B (2008) Light-activated channels targeted to ON bipolar cells restore visual function in retinal degeneration. Nat Neurosci 11:667–675

Lagutin O, Zhu CC, Furuta Y, Rowitch DH, McMahon AP, Oliver G (2001) Six3 promotes the formation of ectopic optic vesicle-like structures in mouse embryos. Dev Dyn 221:342–349

Lagutin OV, Zhu CC, Kobayashi D, Topczewski J, Shimamura K, Puelles L, Russell HR, McKinnon PJ, Solnica-Krezel L, Oliver G (2003) Six3 repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. Genes Dev 17:368–379

Lai K, Kaspar BK, Gage FH, Schaffer DV (2003) Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nat Neurosci 6:21–27

Lamb TM, Knecht AK, Smith WC, Stachel SE, Economides AN, Stahl N, Yancopolous GD, Harland RM (1993) Neural induction by the secreted polypeptide noggin. Science 262:713–718

Lamba DA, Gust J, Reh TA (2009) Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in crx-deficient mice. Cell Stem Cell 4:73–79

Lamba DA, McUsic A, Hirata RK, Wang PR, Russell D, Reh TA (2010) Generation, purification and transplantation of photoreceptors derived from human induced pluripotent stem cells. PLoS One 5:e8763

Lee HY, Wroblewski E, Philips GT, Stair CN, Conley K, Reedy M, Mastick GS, Brown NL (2005) Multiple requirements for Hes 1 during early eye formation. Dev Biol 284:464–478

Lie DC, Colamarino SA, Song HJ, Desire L, Mira H, Consiglio A, Lein ES, Jessberger S, Lansford H, Dearie AR, Gage FH (2005) Wnt signalling regulates adult hippocampal neurogenesis. Nature 437:1370–1375

Lister R, Pelizzola M, Kida YS, Hawkins RD, Nery JR, Hon G, Antosiewicz-Bourget J, O'Malley R, Castanon R, Klugman S et al (2011) Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature 471:68–73

Liu X, Wang Q, Haydar TF, Bordey A (2005) Nonsynaptic GABA signaling in postnatal subventricular zone controls proliferation of GFAP-expressing progenitors. Nat Neurosci 8:1179–1187

Liu GH, Suzuki K, Qu J, Sancho-Martinez I, Yi F, Li M, Kumar S, Nivet E, Kim J, Soligalla RD et al (2011) Targeted gene correction of laminopathy-associated LMNA mutations in patient-specific iPSCs. Cell Stem Cell 8:688–694

Lledo PM, Alonso M, Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci 7:179–193
Lois C, Alvarez-Buylla A (1994) Long-distance neuronal migration in the adult mammalian brain. Science 264:1145–1148
Lois C, Garcia-Verdugo JM, Alvarez-Buylla A (1996) Chain migration of neuronal precursors. Science 271:978–981
Lund RD, Wang S, Klimanskaya I, Holmes T, Ramos-Kelsey R, Lu B, Girman S, Bischoff N, Sauve Y, Lanza R (2006) Human embryonic stem cell-derived cells rescue visual function in dystrophic RCS rats. Cloning Stem Cells 8:189–199
Luo L, Callaway EM, Svoboda K (2008) Genetic dissection of neural circuits. Neuron 57:634–660
MacLaren RE, Pearson RA, MacNeil A, Douglas RH, Salt TE, Akimoto M, Swaroop A, Sowden JC, Ali RR (2006) Retinal repair by transplantation of photoreceptor precursors. Nature 444:203–207
Madsen TM, Kristjansen PE, Bolwig TG, Wortwein G (2003) Arrested neuronal proliferation and impaired hippocampal function following fractionated brain irradiation in the adult rat. Neuroscience 119:635–642
Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yachechko R, Tchieu J, Jaenisch R et al (2007) Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell 1:55–70
Masse K, Bhamra S, Eason R, Dale N, Jones EA (2007) Purine-mediated signalling triggers eye development. Nature 449:1058–1062
Mathers PH, Grinberg A, Mahon KA, Jamrich M (1997) The Rx homeobox gene is essential for vertebrate eye development. Nature 387:603–607
Mazzoni F, Novelli E, Strettoi E (2008) Retinal ganglion cells survive and maintain normal dendritic morphology in a mouse model of inherited photoreceptor degeneration. J Neurosci 28:14282–14292
Merkle FT, Mirzadeh Z, Alvarez-Buylla A (2007) Mosaic organization of neural stem cells in the adult brain. Science 317:381–384
Mirescu C, Gould E (2006) Stress and adult neurogenesis. Hippocampus 16:233–238
Mizuseki K, Sakamoto T, Watanabe K, Muguruma K, Ikeya M, Nishiyama A, Arakawa A, Suemori H, Nakatsuji N, Kawasaki H et al (2003) Generation of neural crest-derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells. Proc Natl Acad Sci U S A 100:5828–5833
Morrison SJ, Kimble J (2006) Asymmetric and symmetric stem-cell divisions in development and cancer. Nature 441:1068–1074
Morrow EM, Furukawa T, Lee JE, Cepko CL (1999) NeuroD regulates multiple functions in the developing neural retina in rodent. Development 126:23–36
Mukhopadhyay M, Shtrom S, Rodriguez-Esteban C, Chen L, Tsukui T, Gomer L, Dorward DW, Glinka A, Grinberg A, Huang SP et al (2001) Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. Dev Cell 1:423–434
Nishida A, Furukawa A, Koike C, Tano Y, Aizawa S, Matsuo I, Furukawa T (2003) Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. Nat Neurosci 6:1255–1263
Niwa H (2007) How is pluripotency determined and maintained? Development 134:635–646
Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448:313–317
Oliver G, Mailhos A, Wehr R, Copeland NG, Jenkins NA, Gruss P (1995) Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. Development 121:4045–4055
Onuma Y, Takahashi S, Asashima M, Kurata S, Gehring WJ (2002) Conservation of Pax 6 function and upstream activation by Notch signaling in eye development of frogs and flies. Proc Natl Acad Sci U S A 99:2020–2025

Ooto S, Akagi T, Kageyama R, Akita J, Mandai M, Honda Y, Takahashi M (2004) Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. Proc Natl Acad Sci U S A 101:13654–13659

Osakada F (2011) Nuclear reprogramming to treat retinal degenerative diseases. Inflamm Regen 31:33–49

Osakada F, Takahashi M (2006) Retinal regeneration by somatic stem cells. Exp Med 24:256–262

Osakada F, Takahashi M (2009) Drug development targeting the glycogen synthase kinase-3beta (GSK-3beta)-mediated signal transduction pathway: targeting the Wnt pathway and transplantation therapy as strategies for retinal repair. J Pharmacol Sci 109:168–173

Osakada F, Takahashi M (2011) Neural induction and patterning in mammalian pluripotent stem cells. CNS Neurol Disord Drug Targets 10:419–432

Osakada F, Ooto S, Akagi T, Mandai M, Akaike A, Takahashi M (2007) Wnt signaling promotes regeneration in the retina of adult mammals. J Neurosci 27:4210–4219

Osakada F, Ikeda H, Mandai M, Wataya T, Watanabe K, Yoshimura N, Akaike A, Sasai Y, Takahashi M (2008) Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. Nat Biotechnol 26:215–224

Osakada F, Ikeda H, Sasai Y, Takahashi M (2009a) Stepwise differentiation of pluripotent stem cells into retinal cells. Nat Protoc 4:811–824

Osakada F, Jin ZB, Hirami Y, Ikeda H, Danjyo T, Watanabe K, Sasai Y, Takahashi M (2009b) In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. J Cell Sci 122:3169–3179

Osakada F, Hirami Y, Takahashi M (2010) Stem cell biology and cell transplantation therapy in the retina. Biotechnol Genet Eng Rev 26:297–334

Osakada F, Mori T, Cetin AH, Marshel JH, Virgen B, Callaway EM (2011) New rabies virus variants for monitoring and manipulating activity and gene expression in defined neural circuits. Neuron 71:617–631

Pfisterer U, Kirkeby A, Torper O, Wood J, Nelander J, Dufour A, Bjorklund A, Lindvall O, Jakobsson J, Parmar M (2011) Direct conversion of human fibroblasts to dopaminergic neurons. Proc Natl Acad Sci U S A 108:10343–10348

Phanstiel DH, Brumbaugh J, Wenger CD, Tian S, Probasco MD, Bailey DJ, Swaney DL, Tervo MA, Bolin JM, Ruotti V et al (2011) Proteomic and phosphoproteomic comparison of human ES and iPS cells. Nat Methods 8:821–827

Polo JM, Liu S, Figueroa ME, Kulalert W, Eminli S, Tan KY, Apostolou E, Stadtfeld M, Li Y, Shioda T et al (2010) Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nat Biotechnol 28:848–855

Rattner A, Nathans J (2006) Macular degeneration: recent advances and therapeutic opportunities. Nat Rev Neurosci 7:860–872

Reh TA, Nagy T (1987) A possible role for the vascular membrane in retinal regeneration in Rana catesbienna tadpoles. Dev Biol 122:471–482

Reh TA, Nagy T, Gretton H (1987) Retinal pigmented epithelial cells induced to transdifferentiate to neurons by laminin. Nature 330:68–71

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710

Reynolds BA, Tetzlaff W, Weiss S (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12:4565–4574

Sanai N, Tramontin AD, Quinones-Hinojosa A, Barbaro NM, Gupta N, Kunwar S, Lawton MT, McDermott MW, Parsa AT, Manuel-Garcia Verdugo J et al (2004) Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature 427:740–744

Sasai Y, Lu B, Steinbeisser H, Geissert D, Gont LK, De Robertis EM (1994) Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell 79:779–790

Sasai Y, Lu B, Steinbeisser H, De Robertis EM (1995) Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. Nature 376:333–336

Schmidt-Hieber C, Jonas P, Bischofberger J (2004) Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature 429:184–187
Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E (2001) Neurogenesis in the adult is involved in the formation of trace memories. Nature 410:372–376
Sun G (2006) Retinal stem/progenitor properties of iris pigment epithelial cells. Dev Biol 289:243–252
Szabo E, Rampalli S, Risueno RM, Schnerch A, Mitchell R, Fiebig-Comyn A, Levadoux-Martin M, Bhatia M (2010) Direct conversion of human fibroblasts to multilineage blood progenitors. Nature 468:521–526
Tada M, Takahama Y, Abe K, Nakatsuji N, Tada T (2001) Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr Biol 11:1553–1558
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
Takahashi M, Palmer TD, Takahashi J, Gage FH (1998) Widespread integration and survival of adult-derived neural progenitor cells in the developing optic retina. Mol Cell Neurosci 12:340–348
Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
Tropepe V, Coles BL, Chiasson BJ, Horsford DJ, Elia AJ, McInnes RR, van der Kooy D (2000) Retinal stem cells in the adult mammalian eye. Science 287:2032–2036
van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2:266–270
Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M (2010) Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463:1035–1041
Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R (1998) Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394:369–374
Wan J, Zheng H, Xiao HL, She ZJ, Zhou GM (2007) Sonic hedgehog promotes stem-cell potential of Muller glia in the mammalian retina. Biochem Biophys Res Commun 363:347–354
Warner-Schmidt JL, Duman RS (2006) Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment. Hippocampus 16:239–249
Watanabe K, Kamiya D, Nishiyama A, Katayama T, Nozaki S, Kawasaki H, Watanabe Y, Mizuseki K, Sasai Y (2005) Directed differentiation of telencephalic precursors from embryonic stem cells. Nat Neurosci 8:288–296
Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MA, Lassar AB, Miller AD (1989) Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci U S A 86:5434–5438
Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448:318–324
Wichterle H, Lieberam I, Porter JA, Jessell TM (2002) Directed differentiation of embryonic stem cells into motor neurons. Cell 110:385–397
Wright AF, Chakarova CF, Abd El-Aziz MM, Bhattacharya SS (2010) Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait. Nat Rev Genet 11:273–284
Yamanaka S (2009) Elite and stochastic models for induced pluripotent stem cell generation. Nature 460:49–52
Young TL, Cepko CL (2004) A role for ligand-gated ion channels in rod photoreceptor development. Neuron 41:867–879
Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R et al (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920
Yusa K, Rashid ST, Strick-Marchand H, Varela I, Liu PQ, Paschon DE, Miranda E, Ordonez A, Hannan NR, Rouhani FJ et al (2011) Targeted gene correction of alpha1-antitrypsin deficiency in induced pluripotent stem cells. Nature 478:391–394
Zuber ME, Gestri G, Viczian AS, Barsacchi G, Harris WA (2003) Specification of the vertebrate eye by a network of eye field transcription factors. Development 130:5155–5167

Part II
Stem Cell Science and Technology

Chapter 6
Characterization and Classification of Stem Cells

Ute Bissels, Dominik Eckardt, and Andreas Bosio

Abstract Starting from a zygote, an organism is made up of thousands, highly organised stem cells, progenitor cells and postmitotic cells which are generated in spatio-temporally coordinated proliferation and differentiation steps. The ongoing advancements in cell culture, isolation techniques, and molecular analyses have driven our basic understanding of different cell types and led to a broad classification of stem cells. This chapter outlines the most prominent techniques used for the characterization and classification of stem cells and provides an overview of many different stem cells, their function and their mRNA, miRNA and protein content.

Abbreviations

ESC	embryonic stem cell
iPSC	induced pluripotent stem cell
HSC	hematopoietic stem cell
TSC	tissue stem cell
CSC	cancer stem cell
EPC	endothelial progenitor cell
SPC	spermtogonial progenitor cell
HpSC	hepatic stem cell
NSC	neural stem cell
BTSC	brain tumor stem cell
MSC	mesenchymal stem cell
LT-HSC	long-term hematopoietic stem cell

U. Bissels, Ph.D. • D. Eckardt, Ph.D. • A. Bosio, Ph.D. (✉)
Miltenyi Biotec GmbH, Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
e-mail: andreas.bosio@miltenyibiotec.de

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_6,

ST-HSC	short-term hematopoietic stem cell
MP	multipotent progenitors
CMP	common myeloid progenitor
CLP	common lymphoid progenitor
MEP	megakaryocyte-erythroid progenitor
GMP	granulocyte-macrophage progenitor
ErP	erythroid progenitor
MkP	megakaryocyte progenitor
RBC	red blood cells
NK	natural killer

6.1 Introduction

The characterization of stem cells helps us to shed light into general cellular processes and to understand the development and senescence of organs and organisms. It is also a prerequisite to use stem cells as tools for drug target discovery, predictive toxicology, or for cellular therapies including tissue regeneration. A classification of stem cells can be done by measuring and quantifying distinct functional properties and/or molecular markers. While the function of self renewal defines stem cells in general, the degree of "potency" i.e. the range of differentiation options to generate different cell types is commonly used for a rough hierarchical classification of cells into:

- totipotent cells: generate all cells including extraembryonic cell types, e.g. zygote
- pluripotent cells: generate all body cells including germ cells, e.g. embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), inner cell mass of the blastocyst-stage embryo
- multipotent cells: generate all tissues cells, e.g. tissue stem cells like hematopoietic stem cells (HSCs)
- unipotent cells: generate a single cell type, e.g. spermatogonial stem cells (SPCs)

The hierarchy is not unidirectional as in certain circumstances a cell can dedifferentiate to form cells with a higher potency.

A further classification subdivides the different multipotent stem cells according to the tissue cells they can generate. It is assumed that almost every tissue has stem cells which are responsible to keep tissue homeostasis and to regenerate or limit injuries. Most prominent multipotent or tissue stem/progenitor cells are those forming the blood (hematopoietic stem cells, HSCs), endothelium (endothelial progenitor cells, EPCs), mesenchyme (mesenchymal stem/stroma cells, MSCs), muscles (satellite stem cells), heart (cardiac stem/progenitor cells), sperm (spermatogonial stem cells), intestine (intestinal stem cells), pancreas (pancreas derived multipotent precursors), lung (lung stem cells), liver (hepatic stem cells), brain (neural stem cells, NSC), skin and hair (skin stem cells), and mammary glands (mammary stem cells).

The borders are not strict as, although rare in vertebrates, a transdifferentiation of one tissue stem cell into another tissue lineage has been reported in vitro and in vivo.

As a certain function of a cell is usually made up by a complex and time dependent interplay of different molecule classes it is occasionally difficult to measure or even to quantify it. That's why a purely functional classification of stem cells is sometimes not of practical help and molecular markers come into play. Technical limitations in terms of sensitivity, specificity and ease of (parallelized) measuring further define which markers or class of markers are eventually used for a certain cell type. Let us take for example the definition of embryonic stem cells, or in general pluripotent stem cells. The term "pluripotent cell" has mainly been derived from the properties of an embryonic stem cell. An embryonic stem cell can give rise to all the cells and tissues of an organism with the exception of the extra embryonic tissue. With this definition it is clear that in order to proof a cell of being pluripotent one has to show that this cell when injected into a blastocyst stage embryo is able to generate a whole organism including the germ cells. This is almost only possible with mice, certainly not with human cells. That is why teratoma formation has been introduced as a surrogate test. Here, the potential of a cell to differentiate into any of the three germ layers: endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system) is interrogated. But even this is very time consuming, not really quantifiable and can not be used as a prospective definition but only as a retrograde proof. Therefore, molecular markers have been defined which are correlated with pluripotency like certain proteins expressed on the surface of pluripotent cells, transcription factors, microRNAs (miRNAs), messenger RNAs (mRNAs) or the methylation status of genomic sequences. Still, after many years, it is hotly debated which are the right pluripotency markers and whether it is acceptable at all to rely only on makers when referring to pluripotency. In conclusion, a classification of stem cells is based on both, molecular markers for practical reasons and their function for reasons of clarity.

6.2 Methods for the Characterization and Classification of Stem Cells

From a biochemical point of view stem cells do not differ from other cells and thus all known methods which allow to measure the status and interaction of biomolecules can be used to characterize stem cells. However, for stem cells the description of some biomolecules using certain techniques has been found to be more instrumental than others.

– DNA methylation: It stably alters the gene expression pattern in cells resembling if a gene is likely to be transcribed (active) or not (silenced). It is measured for instance by Methylation Specific PCR (MSP), or ChIP-on-chip assays.

- mRNA status or transcriptome: It tells which genes are transcribed and are therefore active. As all transcripts in a cell can be measured in parallel using microarrays or library sequencing, a good estimation of all active genomic pathways can be drawn.
- miRNAs: they are analysed like mRNAs using PCR, blotting techniques, microarrays, sequencing, and in situ hybridisation and are a relatively young class of molecules which help to understand if corresponding mRNAs are translated to proteins or not. Their expression has been found to be quite robustly correlated to some cell types.
- Cell surface molecules: They can be identified mainly by their reaction with specific antibodies using techniques like flow cytometry, immunohistochemistry, immunocytochemistry, or different sorts of gel electrophoresis and blotting. In addition mass spectrometry is used to analyse the cell surface proteome without antibodies. Also, raising new antibodies by immunisation of rats and mice with cells has led to the identification of many new markers. Especially adhesion molecules and receptors can also be analysed using the respective interaction partners and give insights into the "communication status" of a cell. The massive advantage of surface proteins or molecules in general is that they can be used to sort cells very easily e.g. by using flow cytometry based sorting, immunopanning, or magnetic cell sorting. In order to standardize the annotation of surface molecules a CD (cluster of differentiation) nomenclature was established in 1982 at the 1st International Workshop and Conference on Human Leukocyte Differentiation Antigens (HLDA). The CD system originally classifies monoclonal antibodies (mAbs) generated against epitopes on the surface of leukocytes and has then been expanded to many other cell types.
- Transcription factors: They are very indicative for some cell types as they resemble which pathways of a cell are activated and which not. Many of them are a master switch deciding which lineage a cell is following. Their importance has been proofed by the fact that the ectopic expression of single transcription factors can redirect (or reprogram) the differentiation fate of a cell.
- Cell surface membrane transporter: At least some stem cells differ from non-stem cells in their ability to transport Hoechst stains (Hoechst 33342) out of the cell. Hoechst 33342 is a DNA-binding fluorescent dye, excitable by ultraviolet light at 350 nm and emitting at 461 nm. A multidrug-like transporter in stem cells causes an increased efflux of Hoechst 33342 by an active biological process. This can be used to identify stem cells by flow cytometry as a "side population" (1996).
- Enzymes: Stem and progenitor cells also possess a different aldehyde dehydrogenase (ALDH) activity compared to other cells. This enzyme converts a non-fluorescent substrate (an aminoacetaldehyde) into a fluorescent product (an aminoacetate) that is retained within living cells with an intact membrane. Cells with different ALDH enzyme activity can thus be differentially stained with the fluorescent product, and stem cells can be isolated by flow cytometry based on their enzyme activity (Jones et al. 1995; Storms et al. 1999).

The analysis of most of the above mentioned molecules is optimally done on highly purified stem cells rather than mixtures of different cell types. A detailed description of techniques for the enrichment of stem cells has recently been reviewed (Bosio et al. 2009).

Interestingly, although it is an absolute prerequisite for single cell-based isolation and characterization of stem cells, we noticed a lack in standardized protocols for proper dissociation of tissues. Solid organs consist of a mixture of cell types which are interconnected in multiple ways. Specific transport proteins as well as gap junctions connect cells and allow for the transport of molecules, whereas tight junctions build up a barrier to avoid free transport across cell layers. In addition, cell adhesion molecules like cadherins are important for stability of the tissue and localization of the cells. All cells in these tissues are surrounded by a complex extracellular matrix composed of a variety of proteins and polysaccharides. The most important components are collagens, hyaluronan, and glycosaminoglycan (Iozzo 1998). The major goal of tissue dissociation is to disrupt the extracellular matrix and cell adhesion components without harming the integrity of the cell membrane and the surface epitopes. We have established automated procedures for the enzymatic and mechanical dissociation of solid tissues and optimized them according to the specific needs of a given tissue or cell type (Jungblut et al. 2008, 2009; Pennartz et al. 2009).

An interesting approach combining the knowledge of stem cell type specific gene expression with the convenience of surface markers is the use of genetically modified stem cells to label or enrich these cells. Here, the promoter of a gene specifically expressed in a cell type is used to drive the expression of a selection marker such as the green fluorescence protein (GFP), an antibiotic resistance gene or an artificial surface epitope like the human CD4 molecule lacking its intracellular domain.

In vitro and in vivo assays to functionally characterize stem cells are partially dependent on the respective stem cell, but some assays are used for multiple stem cell types. For example, measuring the replication of cells by incorporating detectable molecules like BrdU into the DNA, or proliferation of cells by CFSE via staining of intracellular proteins. This allows to distinguish non dividing (postmitotic) cells from proliferating or differentiating ones. In vitro culturing and differentiation of cells as well as the transplantation of cells into animal models are methods used to track the differentiation potential, the regenerative power or malignancy of stem cells. Culturing of stem cells in semi-solid media (colony forming unit (CFU) assays) offers the opportunity to analyse the lineages and to quantify the number of colonies derived from stem cells and is especially used for hematopoietic stem cells.

6.3 Protein Markers of Stem Cells

Protein markers are widely used for classification of stem cells. This is due to the fact that the expression of proteins is less variable than for example mRNA expression and that, especially for proteins expressed on the cell surface, it is possible to use them for the isolation of the respective cells by e.g. immunopanning, flow cytometric sorting, or magnetic sorting. Once the cells are isolated they can be further analysed which allows a clear decision to which extend a protein marker is reflecting a stem cell function. Figures 6.1 and 6.2 summarize the most commonly used markers

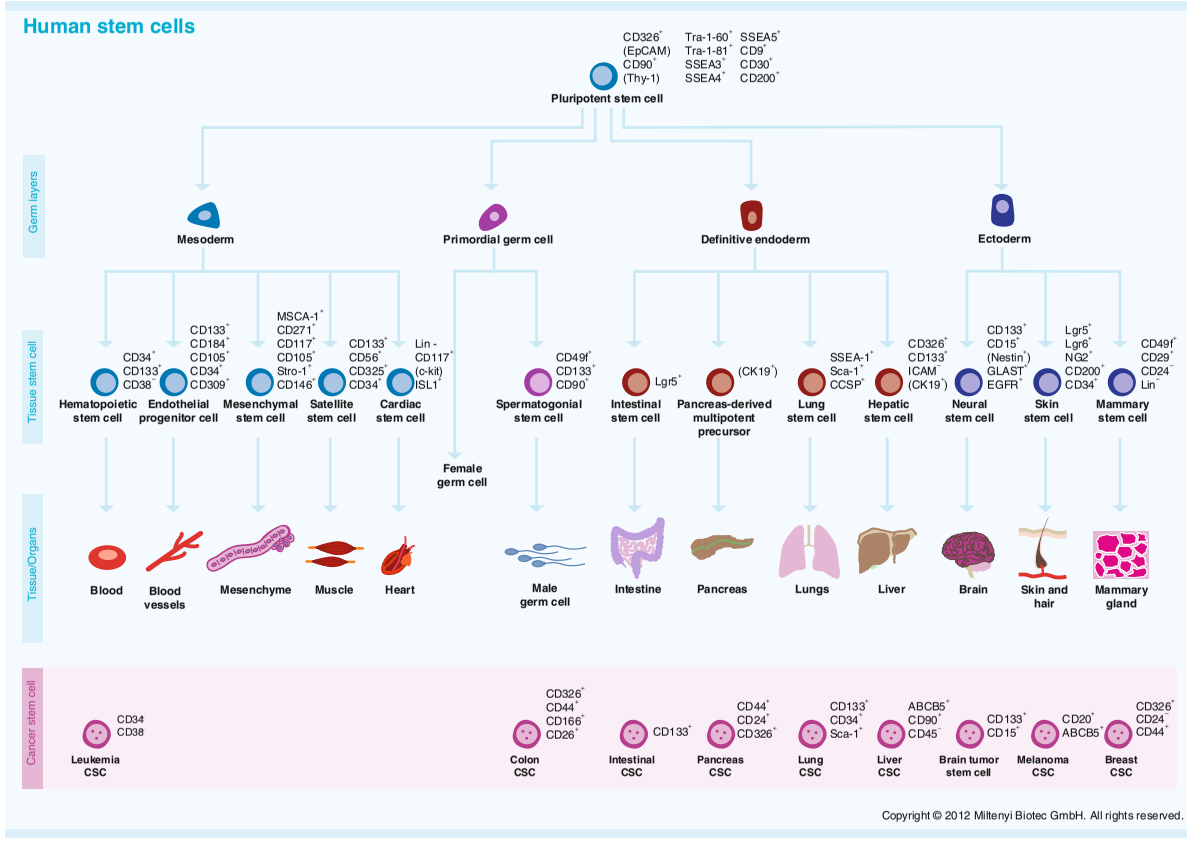


Fig. 6.1 Hierarchical illustration of human stem cells and their cell surface markers

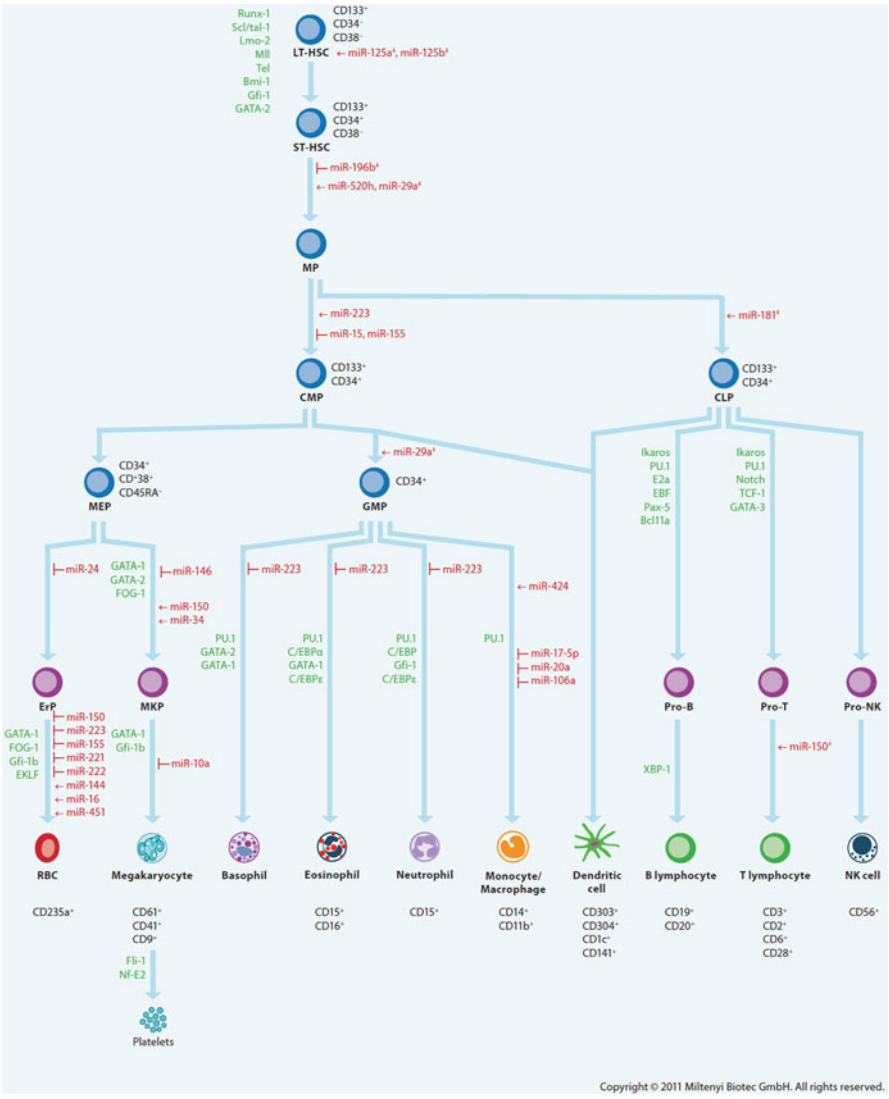


Fig. 6.2 Prominent miRNAs, transcription factors and cell surface markers in hematopoiesis. The miRNAs that regulate the different steps of hematopoiesis are shown in *red*. The depicted miRNAs were mainly identified in in vitro assays with human cells. The role of the miRNAs labelled with ‡, e.g. miR-181‡ that drives differentiation towards CLPs, were identified in mouse experiments. The transcription factors are selected according to Orkin and Zon (2008). Abbreviations: *LT-HSC* long-term hematopoietic stem cell, *ST-HSC* short-term hematopoietic stem cell, *MP* multipotent progenitors, *CMP* common myeloid progenitor, *CLP* common lymphoid progenitor, *MEP* megakaryocyte-erythroid progenitor, *GMP* granulocyte-macrophage progenitor, *ErP* erythroid progenitor, *MkP* megakaryocyte progenitor, *RBC* red blood cells, *NK* natural killer

for the different types of human stem/progenitor and cancer stem cells. Like the cells which make up a tissue, tumor cells are functionally heterogeneous. They are organized in a hierarchy of cell populations with different biological properties. Only a minority of tumor cells have the capacity to regenerate a tumor and sustain its growth when injected into an immune-compromised mouse model which is the functional definition of a cancer stem cell (Tang et al. 2007).

For mouse **pluripotent cells** such as ESCs and iPSCs, mainly E-cadherin (CD324), EpCAM (CD326) and SSEA-1 (CD15) have been used as surface marker. Different proteomic strategies like mass spectrometry of mouse ESCs revealed further details about the cell surface signature of pluripotent mouse stem cells (Nunomura et al. 2005; Wollscheid et al. 2009). Mostly EpCAM (CD326), E-cadherin (CD324), CD90, SSEA-3, SSEA-4, SSEA-5, CD9, TRA-1-60, and TRA-1-81 have been used to characterize human ESCs and iPSCs (Adewumi et al. 2007; Tang et al. 2007). Interestingly, the carbohydrate SSEA-1 is a pluripotency marker in case of mouse pluripotent stem cells, in the human system, SSEA-1 is indicative of pluripotent stem cell differentiation. More than 200 cell surface proteins of the human embryonic stem cell line HUES-7 have been identified by Dormeyer et al. (2008).

Murine **hematopoietic stem and progenitor cells**, HSCs, have been defined by absence of lineage commitment markers such as CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), and Ter-119, and high expression of CD117 (c-kit/SCFR) and Sca-1 (Hubin et al. 2005; Schiedlmeier et al. 2007). CD34 is expressed on HSCs of the murine fetus and neonate, but decreases with age (Ogawa 2002). Another way of defining hematopoietic stem and progenitor cells is the use of SLAM markers (Kiel et al. 2005). Accordingly, multipotent HSCs are CD150+CD48−CD244−, multipotent progenitor cells (MPPs) are CD150−CD48−CD244+, and lineage-restricted progenitor cells (LRPs) are CD150−CD48+CD244+. CD34 and CD133 label human HSCs with long-term engraftment in NOD/SCID mice. However, about 95% of the CD34+ cells and 70% of the CD133+ cells have a progenitor status, identified by co-expression of CD38. Therefore, CD34+CD38−CD133+ is mostly used as the surface signature of human HSCs (Buhring et al. 1999; Copland et al. 2006; Giebel et al. 2006). John Dick and colleagues isolated and identified CD34+CD38− **leukemic stem cells** (LSCs) from human AML by FACS and demonstrated that these cells initiated leukemia in NOD-SCID mice compared with the CD34+CD38+ and CD34− fractions (Bonnet and Dick 1997). An engrafted leukemia could be serially transplanted into secondary recipients, providing functional evidence for self-renewal. Xenotransplantation, followed by serial transplantation, is now regarded as an essential criterion in defining cancer stem cells. The ability to recapture tumor pathophysiology is also an important defining functional criterion of cancer stem cells prospectively isolated (Tang et al. 2007).

Another stem cell type which is found in the bone marrow and mobilized to the blood stream by environmental stimuli for physiological and pathological tissue regeneration are the **endothelial progenitor cells** (EPCs) which form new blood vessels and contribute to vascular repair (Asahara et al. 2011). In humans, these cells have been defined by the expression of the markers CD34, CD133, CD309 (VEGFR2/KDR/Flk-1), CD184 (CXCR4), CD105 (Endoglin), and in the mouse by

Lin− Sca-1+ c-kit+ CD34+ CD309+ (VEGFR-2/KDR/Flk-1) (Rafii and Lyden 2003; Timmermans et al. 2009). Nevertheless, the identification of a unique combination of receptors specific and selective for primary EPCs, enabling an unambiguous distinction between EPCs and HSCs is still missing.

Several cell surface antigens have been suggested for the isolation of **mesenchymal stem/stromal cells**, MSCs, such as antifibroblast antigen (Jones et al. 2002), CD117 (Huss and Moosmann 2002), CD105 (Majumdar et al. 2003; Aslan et al. 2006), Stro-1 and CD146 (Shi and Gronthos 2003), CD133 (Tondreau et al. 2005), CD271 (Quirici et al. 2002) and MSCA-1 (W8B2) (Buhring et al. 2007). A comprehensive cell surface proteome analysis of human plastic adherent MSCs has been published recently by Niehage et al. (2011), describing even among the 41 identified CD markers, 5 epitopes previously not linked to the MSC cell surface. MSCs expanded from mouse bone marrow culture are described to be positive for Sca-1, CD117 (c-kit), and CD105 (Sun et al. 2003).

Neural stem cells (NSCs) share many characteristics with astrocytes and show expression of typical astrocyte proteins, like GFAP, or GLAST (Mori et al. 2005; Merkle and Alvarez-Buylla 2006). Furthermore, CD133/Prominin, EGF receptor, CD15, and Nestin have been described as markers for neural stem cells (Conti and Cattaneo 2010), but isolation of these cells from primary neural tissue with high purity has been difficult. Therefore, a combination of markers has been used to increase the purity. Beckervordersandforth et al. (2011) followed a dual labeling strategy to isolate GFAP/prominin1 double positive self-renewing multipotent stem cells from adult hGFAP-GFP mice in combination with prominin labeling. In another approach GFAP/EGFR+ cells were successfully isolated and identified as activated stem cell astrocytes (Pastrana et al. 2009). Many more cell surface proteins have been described and used for sorting of **neural progenitor cells** like PSA-NCAM (neuronal precursors) (Boutin et al. 2010; Pennartz et al. 2004), and A2B5 (glial precursors) (Seidenfaden et al. 2006). Singh et al. (2003, 2004a) reported the identification and purification of **cancer stem cells** from human brain tumors of different phenotypes that possess a marked capacity for proliferation, self-renewal, and differentiation.

The increased self-renewal capacity of the **brain tumor stem cell** (BTSC) was highest among the most aggressive clinical samples of medulloblastoma compared with low-grade gliomas. Several other reports demonstrated that isolation of cells expressing the surface marker CD133 leads to enrichment of the BTSC population (Singh et al. 2004b; Bao et al. 2006; Piccirillo and Vescovi 2006), whereas Son et al. (2009) showed that SSEA-1 (CD15) enriches for tumorigenic subpopulations in human Glioblastoma.

Existence of various resident populations of **cardiac progenitor/stem cells** in postnatal hearts has been claimed (Sturzu and Wu 2011). CD117 (c-kit)+/lin– cells isolated from the adult mouse heart appeared to be clonogenic and self-renewing, capable of differentiating into cardiomyocytes, vascular smooth muscle cells, and endothelial cells (Beltrami et al. 2003). Nevertheless, this population only heterogenously expresses early cardiac transcription factors such as GATA4, Mef2c, and Nkx2.5. Two other publications (Oh et al. 2003; Pfister et al. 2005) referred to the

Sca-1+ population as putative adult cardiac progenitors. Expression of early cardiac transcription factors GATA4 and Mef2c, as well as telomerase activity, associated with self renewal potential, were detected in Sca-1+ cells. However, in contrast to data from transplanted CD117+ cells, fusion between Sca-1+ cells and host cardiomyocytes was frequently detected, leaving some uncertainty about the true *in vivo* differentiation potential of Sca-1+ progenitors (Oh et al. 2003). Expression of the transcription factor Isl-1 in multipotent heart progenitors found in fetal mouse and human heart has not yet been correlated with a distinct surface marker which would allow for antibody-based enrichment (Bu et al. 2009). As described in mice, a CD117+ population of cardiac cells has been found in the human heart exhibiting key characteristics of stem cells: self-renewal, clonogenicity, and multipotency *in vitro* and *in vivo* (Bearzi et al. 2007). In addition, several groups have described *in vitro* cardiomyogenic potential of cardiac cells reactive to an antibody against the mouse Sca-1 epitope. Lastly, a heterogenous cell population isolated from human heart biopsies forms so called cardiospheres in suspension culture. Cardiosphere containing CD117+, CD133+, CD105+ and CD90+ cells have as well been ascribed stem cell characteristics (Smith et al. 2007). To date there is no consensus on the best marker (set) for unambiguous identification of cardiac stem cells.

Several surface markers have been described and used for isolation of mouse **spermatogonial stem cells** (SSC). In 2004 Kubota (Kubota et al. 2004) described a Thy-1 (CD90) antibody-based enrichment of mouse SSCs, further expansion on STO feeder cells in serum-free medium and *in vivo* proof of an SSC phenotype after transplantation. Seandel et al. (2007) showed that SPCs express GPR125, an orphan adhesion-type G-protein-coupled receptor, and can be efficiently obtained by cultivation on mitotically inactivated testicular feeders containing CD34+ stromal cells. Recently, Kanatsu-Shinohara et al. (2011) showed that SSCs have an unstable side population phenotype and provide evidence that SSCs change their phenotype characteristics in response to their microenvironment. A study by Conrad et al. (2008) described the isolation and characterization of human germline stem cells (GSCs) using defined cultivation techniques, SPC adhesion properties and a positive selection using CD49f, CD133, or CD90.

According to Schmelzer et al., **human hepatic stem cells** (hHpSCs) (Schmelzer et al. 2007; Schmelzer and Reid 2008) can be isolated by positive immunoselection for the epithelial cell adhesion molecule CD326 (EpCAM+). The hHpSCs express cytokeratins 7 and 19, CD133, telomerase, CD44H, claudin 3, and albumin (weakly). They are negative for alpha-fetoprotein (AFP), intercellular adhesion molecule 1 (ICAM-1), and for markers of adult liver cells (cytochrome P450s) and hematopoietic (progenitor) cells (CD45, CD34, CD14, CD38, CD90 (Thy-1), CD235a (Glycophorin A)). As for rodent HpSCs, Yovchev et al. compared hepatic cells isolated by two surface markers, EpCAM and Thy-1 (CD90). It was shown that Thy-1+ cells are mesenchymal cells with characteristics of myofibroblasts/activated stellate cells while transplantation experiments revealed that EpCAM+ cells are true progenitors capable of repopulating injured rat liver (Yovchev et al. 2007, 2008).

Yang et al. (2008) have delineated **liver cancer stem cells** serially from HCC cell lines, human liver cancer specimens, and blood samples, using CD90 as a marker. CD45− CD90+ cells were detected in all the tumor specimens, but not in the normal,

cirrhotic, and parallel non-tumorous livers. Cheung et al. (2011) have shown that expression of ABCB5 (ATP-dependent binding cassette B5) in liver cancer stem cells is associated with chemoresistance and reduced survival times of patients with hepatocellular carcinoma. **Mammary stem cells** have been characterized by the markers CD49f, CD29 (also known as α6 and β1 integrins) and CD24 when showing a CD24lowCD49f high or CD24lowCD29high molecular signature (Shackleton et al. 2006; Stingl et al. 2006). In contrast to their differentiated progeny, mammary stem cells are negative for estrogen receptor (ERα), progesterone receptor (PR) and the tyrosine kinase receptor HER2 – three molecular markers that define different populations of differentiated luminal epithelial cells – but are highly positive for the transcription factor p63, the epidermal growth factor receptor (EGFR) and cytokeratin 14 (CK14), confirming their basal origin (Asselin-Labat et al. 2006; Pontier and Muller 2009). **Breast cancer stem cells** have been reported to be ESA+CD44+CD24−Lineage− (Al-Hajj et al. 2003). ESA (epithelial specific antigen) is also known as EpCAM (CD326). O'Brien et al. (2007) and Ricci-Vitiani et al. (2007) showed that the tumorigenic population in **colon cancer** is restricted to CD133+ cells, which are able to reproduce the original tumor in permissive recipients. Additionally, the surface marker pattern CD326 (EpCAM)+CD44+ CD166+ has been described by Du et al. (2008) and Dalerba et al. (2007). Pang et al. (2010) have described CD26 as marker for the tumorigenic population in colon cancer.

Li et al. (2007) identified a highly tumorigenic subpopulation of **pancreatic cancer cells** expressing the cell surface markers CD44, CD24, and epithelial- specific antigen (ESA; EpCAM; CD326). Pancreatic cancer cells with the CD44+CD24+ESA+ phenotype (0.2–0.8% of pancreatic cancer cells) had a 100-fold increased tumorigenic potential compared with non-tumorigenic cancer cells, with 50% of animals injected with as few as 100 CD44+CD24+ESA+ cells forming tumors that were histologically indistinguishable from the human tumors from which they originated.

As a conclusion, protein markers correlated to functional properties of the respective stem/progenitor cell types have been defined for most tissues and pluripotent cells. However, some of the markers have only recently been reported and are still intensively debated. It can be estimated that sorting of pluripotent and tissue stem cells will increase in the future as it offers the option for a detailed analysis and understanding of malignant and disease-causing cells, as well as of cell types urgently needed for tissue regeneration and tissue engineering approaches.

6.4 miRNAs in Stem Cells

MicroRNAs (miRNAs), short noncoding RNAs of 21–23-nucleotides (nt) in length, regulate target mRNAs post-transcriptionally. miRNAs in stem cells are not as well characterized as proteins. However, they have been shown to play an important role in many different cellular, developmental, and physiological processes as divergent as cell lineage decisions, cell proliferation, apoptosis, morphogenesis, fat metabolism, hormone secretion, neuronal synaptic plasticity, and long-term memory (Aravin and Tuschl 2005).

In 2004, it was shown for the first time that miRNAs are involved in hematopoietic lineage differentiation (Chen et al. 2004). For example, ectopic expression of miR-181 in lineage negative (Lin-) hematopoietic progenitor cells from mouse bone marrow increased the fraction of B-lineage cells (CD19+) in vitro and in vivo. As summarized in Fig. 6.2, further analysis showed that miRNAs fine tune essentially each step in hematopoiesis. It was demonstrated, for instance, that miR-150 drives megakaryocyte-erythrocyte progenitor (MEP) differentiation towards megakaryocytes at the expense of erythroid cells (Lu et al. 2008). Erythropoiesis was reported to be promoted by miR-451, miR-16 and miR-144 and negatively regulated by miR-150, miR-155, miR-221, miR-222 and miR-223 (Felli et al. 2005; Bruchova et al. 2007; Zhan et al. 2007; Dore et al. 2008). Furthermore, it was shown that the miRNA cluster miR-17-5p-92 controls monocytopoiesis (Fontana et al. 2007) and that miR-424 is upregulated during monocyte/macrophage differentiation. Within the lymphoid lineage, the decision between T cells and B cells is regulated by miR-150 (Xiao et al. 2007; Zhou et al. 2007).

The early steps of HSC differentiation, e.g. the role of miRNAs in self-renewal of the LT-HSCs and ST-HSC as well as the function of miRNAs in mulipotent progenitors, are currently mostly unknown due to the difficulty to perform whole genome miRNA screens of small numbers of cells. Up to now, expression of miRNAs was analysed in human primitive Lin-CD34+CD38-CD90+CD45RA- cells (Han et al. 2010; Ooi et al. 2010), CD34+CD38– cells (Liao et al. 2008), CD133+ cells (Jin et al. 2008; Bissels et al. 2011b) and murine HSCs (Guo et al. 2010; O'Connell et al. 2010; Petriv et al. 2010). Liao and coworkers found miR-520h to be overexpressed in CD34+CD38– cells compared to more commited CD34+ cells. Ooi et al. (2010) compared HSCs (Lin–CD34+CD38–CD90+CD45RA–) and MPPs (Lin–CD34+CD38–CD90–CD45RA–) to more committed progenitor populations and found miR-125b to be highly expressed in the stem cell fractions. Recently, we presented the first relative and absolute miRNA copy number profile of CD133+ bone marrow cells and directly compared donor-matched CD133+ cells with the more differentiated CD34+CD133- and CD34-CD133- cells on miRNA and mRNA level (Bissels et al. 2009, 2011b). 18 miRNAs were significantly differentially expressed between CD133+ and CD34+CD133- cells. These differentially expressed miRNAs are involved in inhibition of differentiation, prevention of apoptosis, and cytoskeletal remodelling. miRNA expression profiles are further available for CD34+ progenitor cells from bone marrow and mobilized peripheral blood (Georgantas et al. 2007) as well as from cord blood (Merkerova et al. 2009). A recent study by Arnold et al. (2011) identified miRNAs shared by multiple tissue-specific stem cells and miRNAs unique to various tissue-specific murine stem cells. miR-192 was identified as specific for LT-HSCs ($Endoglin^{+}Rho^{low}Sca\text{-}1^{+}Lin^{-}$) and absent from all other analysed cell types.

While the different cell types of the hematopoietic system express a multitude of miRNAs, five were reported to be common hematopoietic miRNAs, namely miR-142, miR-144, miR-150, miR-155 and miR-223. Those miRNAs were identified as highly specific for hematopoietic cells within a large-scale study to identify miRNAs and to assess their expression patterns in >250 small RNA libraries from >26 different organ systems (Landgraf et al. 2007).

Specifically expressed miRNAs are also known for other types of stem cells e.g. cancer stem cells (CSCs) and human embryonic stem cells (hESCs). Breast cancer stem cells (BCSCs) are characterized among others by downregulation of miR-200c. Importantly, miR-200c suppresses tumorigenicity of BCSCs (Shimono et al. 2009). In hESCs the miR-302~367 cluster is specifically expressed (Suh et al. 2004; Landgraf et al. 2007) and may therefore serve as a marker for hES cells. The first miRNA profile of induced pluripotent stem cells (iPSC) revealed that the miR-302~367 cluster is also highly expressed in the reprogrammed cells (Wilson et al. 2009). Recently, it has been shown that expression of the miR302/367 cluster can directly reprogram mouse and human somatic cells to a pluripotent stem cell state in the absence of the commonly used transcription factors Oct4, Sox2, Klf4 and Myc (Anokye-Danso et al. 2011). This miRNA-based reprogramming approach is two orders of magnitude more efficient than standard methods. Miyoshi et al. (2011) showed that reprogramming of murine and human cells is even feasible by direct transfection of mature miRNAs with a non-viral approach. Taken together, the characterization of stem cells with respect to miRNAs is well advanced for some stem cell types and has almost not been addressed for some other stem cell and progenitor cell types. This is partly due to difficulties to isolate enough stem cells for a proper miRNA analyses and it is likely to be solved in the next years. Only then it will, if at all, be possible to speculate on common miRNA signatures of stem cells and to shed light into the miRNA based regulation of stem cell related cellular functions. For further reading about the role of miRNAs in stem cells, we recommend the following reviews: Hatfield and Ruohola-Baker (2008), Gangaraju and Lin (2009), Mallanna and Rizzino (2010), Bissels et al. (2011a).

6.5 The mRNA of Stem Cells

In 2002, two independent studies (Ivanova et al. 2002; Ramalho-Santos et al. 2002) tried to identify a general stem cell signatures by comparing the expression profiles of embryonic, hematopoietic and neural stem cells. The two lists of "stemness" enriched transcripts however yielded only 15 common genes (Burns and Zon 2002) which was kind of disappointing. Later on, a third independent expression profiling study (Fortunel et al. 2003) reduced the list of commonly expressed genes to just one: integrin alpha-6. Thus, a universal stem cell signature may not exist, but each stem cell type may have its own transcriptional network responsible for certain unique stem cell properties (Gerrits et al. 2008). A comprehensive transcriptome analysis of human hematopoiesis was recently carried out by Novershtern et al. (2011) and revealed dense transcriptional circuits in HSCs, that gradually disappear during differentiation, while new but less intricate circuits emerge.

With respect to hematopoietic stem cells, a lot of gene expression profiling studies have been carried out. Most of them compared either CD34+ CD38– Lin– cells with CD34+ CD38+ Lin+ cells (Ivanova et al. 2002; Georgantas et al. 2004) or CD133+ with CD133– cells (He et al. 2005; Toren et al. 2005; Hemmoranta

et al. 2006; Jaatinen et al. 2006). Theses studies revealed a number of transcripts overexpressed in HSCs, such as CD133, CD34, the RNA processing protein RBPMS and the receptor tyrosine kinase c-kit. Furthermore, transcription factors as Gata-2, Gata-3, ERG and HLF are overrepresented in HSCs. The transcript BAALC, whose function is unknown, is highly enriched in CD133+ cells (Baldus et al. 2003; Jaatinen et al. 2006). The homolog of the *Drosophila* Dlg1 tumor suppressor gene Dlg7 was identified as a potential stem cell gene by Gudmundsson et al. (2007). However, although the described transcripts have been found as overrepresented in HSCs in most of the studies, it is difficult to name specific mRNA markers for HCSs. The reasons are among others the variability of gene expression profiles due to varying stem cell sources, e.g. BM, CB, and PB (Steidl et al. 2002; Ng et al. 2004), and donor age (Rossi et al. 2005; Nijnik et al. 2007). Table 6.1 summarizes the mRNAs found in hematopoietic stem and progenitor cells.

6.6 Conclusion and Future Developments

The characterization of stem cells is currently rapidly moving forward. While some stem cells like HSCs are already routinely used in clinical settings, many new stem cells have just been described in the last years and many more will be defined in the near future.

Although molecular markers have been named for most of the stem cells, it is also true that many of these markers are not exclusive and certainly not highly specific with respect to a distinct function. This points to essentially three major tasks which need to be addressed: First, a better classification of stem cells with respect to robust molecular markers and especially those markers which can be used for purification of cells. This goes along with technical improvements of sorting techniques, culturing protocols and, moreover, highly sensitive molecular analysis tools. It is challenging as the nature of stem cells includes that they are proliferating slowly and that the cell numbers are small.

Second, a harmonisation of markers and isolation procedures, following the example of the CD nomenclature in the field of immunology. This should improve the exchange and gathering of data about stem cells, which is needed before more stem cell types are entering clinical applications. Third, we need a better understanding of stem cells with respect to their regenerative potential. The reports about reprogramming, dedifferentiation and transdifferentiation of cells and stem cells have raised the notion that essentially all cells can be engineered to generate every type of tissue. This is appealing from a research point of view but raises also some concerns about the predictability of stem cell differentiation when used for tissue regeneration or cellular therapies in general. Solving these issues will broaden our understanding in the exciting field of stem cell biology.

Table 6.1 mRNAs overrepresented in human HSCs

Publication	Ivanova	Georgantas	Jaatinen	Hemmoranta	Toren	Huang	He	Wagner	Wagner
Stem cell fraction	CD34+ CD38-Lin-		CD133+			CD133+	CD133+ CD34+	CD34+ CD38–	CD34+ CD38– SDF
Control fraction	Lin+		CD133–			MSCs, NSCs	CD133–CD34–	CD34+ CD38+	CD34+ CD38– FDF
CD133	–	–	x	x	x	x	x	–	x
RBPMS	x	x	x	–	x	–	x	x	–
CD34	x	–	x	x	x	x	x	–	–
KIT	–	–	x	x	x	x	x	–	–
Gata2	x	–	x	–	x	x	x	–	–
FLJ14054	–	x	x	–	x	–	x	–	x
SPINK2	–	x	x	x	x	–	x	–	–
NRIP1	x	x	x	–	x	–	x	–	–
HOXA9	x	–	x	–	x	–	x	–	x
MEIS1	x	–	x	–	x	x	x	–	–
FHL1	–	–	x	–	x	–	x	–	x
KIAA0125	–	x	x	–	x	–	–	–	x
SOCS2	x	x	x	–	x	–	–	–	–
MLLT3	–	x	x	–	x	x	–	–	–
PLS3	x	x	x	–	x	–	–	–	–
TFP1	x	x	x	–	x	–	–	–	–
ERG	–	x	x	–	x	–	–	–	x
HLF	x	x	–	–	–	x	–	–	–
GUCY1A3	–	x	x	–	x	–	–	–	–
HLF	–	x	x	–	x	–	–	–	–
NPR3	–	x	x	–	x	–	–	–	–
SEPP1	–		x	–	x	–	–	–	–
H2BFQ	–	x	–	–	–	–	x	x	–
GUCY1B3	–	x	–	–	–	–	x	–	x

(continued)

Table 6.1 (continued)

Publication	Ivanova	Georgantas	Jaatinen	Hemmoranta	Toren	Huang	He	Wagner	Wagner
Stem cell fraction	CD34+	CD38-Lin-	CD133+			CD133+	CD133+ CD34+	CD34+ CD38–	CD34+ CD38– SDF
Control fraction	Lin+		CD133–			MSCs, NSCs	CD133-CD34–	CD34+ CD38+	CD34+ CD38– FDF
BAALC	–	–	x	–	x	x	–	–	–
TIE	x	–	–	–	x	–	–	–	–
FLT3	x	–	–	–	x	x	–	–	–
MPL	x	x	–	–	x	–	–	–	–
EVI1	x	x	–	–	x	–	–	–	–
LAPTM4B	x		x	–	x	–	–	–	–
GATA3	x	x	–	–	–	–	–	–	–
HOXB6	x	x	–	–	–	–	–	–	–
MDS1	x	x	–	–	–	–	–	–	–
TRAIL	x	x	–	–	–	–	–	–	–
CEBPB	x	x	–	–	–	–	–	–	–
HOXA3	–	x	x	–	–	–	–	–	–
KIAA1102	–	x	x	–	–	–	–	–	–
D2S448	–	–	x	–	–	–	–	–	x
PLCB1	–	–	x	–	–	–	–	–	x
CRFBP	–	x	–	–	–	–	x	–	–
HIF2	–	x	–	–	–	–	x	–	–
H2A	–	x	–	–	–	–	x	–	–
H2AFO	–	x	–	–	–	–	x	–	–
NPIP1	–	x	–	–	–	–	x	–	–
FZD6	–	–	–	–	x	–	–	–	x
C17	–	–	x	–	x	–	–	–	–
HOXA5	x	–		–	x	–	–	–	–
PKD2	–	x	–	–	x	–	–	–	-

The table encompasses the following publications: Ivanova et al. (2002), Georgantas et al. (2004), Jaatinen et al. (2006), Hemmoranta et al. (2006), Toren et al. (2005), Huang et al. (2008), He et al. (2005), and Wagner et al. (2004)

References

Adewumi O, Aflatoonian B, Ahrlund-Richter L et al (2007) Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol 25:803–816

Al-Hajj M, Wicha MS, Benito-Hernandez A et al (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100:3983–3988

Anokye-Danso F, Trivedi CM, Juhr D et al (2011) Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell Stem Cell 8:376–388

Aravin A, Tuschl T (2005) Identification and characterization of small RNAs involved in RNA silencing. FEBS Lett 579:5830–5840

Arnold CP, Tan R, Zhou B et al (2011) MicroRNA programs in normal and aberrant stem and progenitor cells. Genome Res 21:798–810

Asahara T, Kawamoto A, Masuda H (2011) Concise review: circulating endothelial progenitor cells for vascular medicine. Stem Cells 29:1650–1655

Aslan H, Zilberman Y, Kandel L et al (2006) Osteogenic differentiation of noncultured immunoisolated bone marrow-derived CD105+ cells. Stem Cells 24:1728–1737

Asselin-Labat ML, Shackleton M, Stingl J et al (2006) Steroid hormone receptor status of mouse mammary stem cells. J Natl Cancer Inst 98:1011–1014

Baldus CD, Tanner SM, Kusewitt DF et al (2003) BAALC, a novel marker of human hematopoietic progenitor cells. Exp Hematol 31:1051–1056

Bao S, Wu Q, McLendon RE et al (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444:756–760

Bearzi C, Rota M, Hosoda T et al (2007) Human cardiac stem cells. Proc Natl Acad Sci USA 104:14068–14073

Beckervordersandforth R, Tripathi P, Ninkovic J et al (2011) In vivo fate mapping and expression analysis reveals molecular hallmarks of prospectively isolated adult neural stem cells. Cell Stem Cell 7:744–758

Beltrami AP, Barlucchi L, Torella D et al (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114:763–776

Bissels U, Wild S, Tomiuk S et al (2009) Absolute quantification of microRNAs by using a universal reference. RNA 15:2375–2384

Bissels U, Bosio A, Wagner W (2011a) MicroRNAs are shaping the hematopoietic landscape. Haematologica 97(2):160–167

Bissels U, Wild S, Tomiuk S et al (2011b) Combined characterization of microRNA and mRNA profiles delineates early differentiation pathways of CD133(+) and CD34(+) hematopoietic stem and progenitor cells. Stem Cells 29(5):847–857

Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3:730–737

Bosio A, Huppert V, Donath S et al (2009) Isolation and enrichment of stem cells. Adv Biochem Eng Biotechnol 114:23–72

Boutin C, Hardt O, de Chevigny A et al (2010) NeuroD1 induces terminal neuronal differentiation in olfactory neurogenesis. Proc Natl Acad Sci USA 107:1201–1206

Bruchova H, Yoon D, Agarwal AM et al (2007) Regulated expression of microRNAs in normal and polycythemia vera erythropoiesis. Exp Hematol 35:1657–1667

Bu L, Jiang X, Martin-Puig S et al (2009) Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. Nature 460:113–117

Buhring HJ, Seiffert M, Bock TA et al (1999) Expression of novel surface antigens on early hematopoietic cells. Ann N Y Acad Sci 872:25–38; discussion 38–29

Buhring HJ, Battula VL, Treml S et al (2007) Novel markers for the prospective isolation of human MSC. Ann N Y Acad Sci 1106:262–271

Burns CE, Zon LI (2002) Portrait of a stem cell. Dev Cell 3:612–613

Chen CZ, Li L, Lodish HF et al (2004) MicroRNAs modulate hematopoietic lineage differentiation. Science 303:83–86

Cheung ST, Cheung PF, Cheng CK et al (2011) Granulin-epithelin precursor and ATP-dependent binding cassette (ABC)B5 regulate liver cancer cell chemoresistance. Gastroenterology 140:344–355

Conrad S, Renninger M, Hennenlotter J et al (2008) Generation of pluripotent stem cells from adult human testis. Nature 456:344–349

Conti L, Cattaneo E (2010) Neural stem cell systems: physiological players or in vitro entities? Nature Rev 11:176–187

Copland M, Hamilton A, Elrick LJ et al (2006) Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. Blood 107:4532–4539

Dalerba P, Dylla SJ, Park IK et al (2007) Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci USA 104:10158–10163

Dore LC, Amigo JD, Dos Santos CO et al (2008) A GATA-1-regulated microRNA locus essential for erythropoiesis. Proc Natl Acad Sci USA 105:3333–3338

Dormeyer W, van Hoof D, Braam SR et al (2008) Plasma membrane proteomics of human embryonic stem cells and human embryonal carcinoma cells. J Proteome Res 7:2936–2951

Du L, Wang H, He L et al (2008) CD44 is of functional importance for colorectal cancer stem cells. Clin Cancer Res 14:6751–6760

Felli N, Fontana L, Pelosi E et al (2005) MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. Proc Natl Acad Sci USA 102:18081–18086

Fontana L, Pelosi E, Greco P et al (2007) MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. Nat Cell Biol 9:775–787

Fortunel NO, Otu HH, Ng HH et al (2003) Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". Science 302:393; author reply 393

Gangaraju VK, Lin H (2009) MicroRNAs: key regulators of stem cells. Nat Rev Mol Cell Biol 10:116–125

Georgantas RW 3rd, Tanadve V, Malehorn M et al (2004) Microarray and serial analysis of gene expression analyses identify known and novel transcripts overexpressed in hematopoietic stem cells. Cancer Res 64:4434–4441

Georgantas RW 3rd, Hildreth R, Morisot S et al (2007) CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control. Proc Natl Acad Sci USA 104:2750–2755

Gerrits A, Dykstra B, Otten M et al (2008) Combining transcriptional profiling and genetic linkage analysis to uncover gene networks operating in hematopoietic stem cells and their progeny. Immunogenetics 60:411–422

Giebel B, Zhang T, Beckmann J et al (2006) Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division. Blood 107:2146–2152

Goodell MA, Brose K, Paradis G et al (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183:1797–1806

Gudmundsson KO, Thorsteinsson L, Sigurjonsson OE et al (2007) Gene expression analysis of hematopoietic progenitor cells identifies Dlg7 as a potential stem cell gene. Stem Cells 25:1498–1506

Guo S, Lu J, Schlanger R et al (2010) MicroRNA miR-125a controls hematopoietic stem cell number. Proc Natl Acad Sci USA 107:14229–14234

Han YC, Park CY, Bhagat G et al (2010) microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. J Exp Med 207:475–489

Hatfield S, Ruohola-Baker H (2008) microRNA and stem cell function. Cell Tissue Res 331:57–66

He X, Gonzalez V, Tsang A et al (2005) Differential gene expression profiling of CD34+ CD133+ umbilical cord blood hematopoietic stem progenitor cells. Stem Cells Dev 14:188–198

Hemmoranta H, Hautaniemi S, Niemi J et al (2006) Transcriptional profiling reflects shared and unique characters for CD34+ and CD133+ cells. Stem Cells Dev 15:839–851

Huang TS, Hsieh JY, Wu YH et al (2008) Functional network reconstruction reveals somatic stemness genetic maps and dedifferentiation-like transcriptome reprogramming induced by GATA2. Stem Cells 26:1186–1201
Hubin F, Humblet C, Belaid Z et al (2005) Murine bone marrow stromal cells sustain in vivo the survival of hematopoietic stem cells and the granulopoietic differentiation of more mature progenitors. Stem Cells 23:1626–1633
Huss R, Moosmann S (2002) The co-expression of CD117 (c-kit) and osteocalcin in activated bone marrow stem cells in different diseases. Br J Haematol 118:305–312
Iozzo RV (1998) Matrix proteoglycans: from molecular design to cellular function. Annu Rev Biochem 67:609–652
Ivanova NB, Dimos JT, Schaniel C et al (2002) A stem cell molecular signature. Science 298:601–604
Jaatinen T, Hemmoranta H, Hautaniemi S et al (2006) Global gene expression profile of human cord blood-derived CD133+ cells. Stem Cells 24:631–641
Jin P, Wang E, Ren J et al (2008) Differentiation of two types of mobilized peripheral blood stem cells by microRNA and cDNA expression analysis. J Transl Med 6:39
Jones R, Barber J, Vala M (1995) Assessment of aldehyde dehydrogenase in viable cells. Blood 85:2742–2746
Jones EA, Kinsey SE, English A et al (2002) Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum 46:3349–3360
Jungblut M, Oeltze K, Zehnter I et al (2008) Preparation of single-cell suspensions from mouse spleen with the gentleMACS Dissociator. J Vis Exp 2008 Dec 11, 22(2):1029. doi:10.3791/1029
Jungblut M, Oeltze K, Zehnter I et al (2009) Standardized preparation of single-cell suspensions from mouse lung tissue using the gentleMACS Dissociator. J Vis Exp 29:e1266
Kanatsu-Shinohara M, Takashima S, Ishii K et al (2011) Dynamic changes in EPCAM expression during spermatogonial stem cell differentiation in the mouse testis. PLoS One 6:e23663
Kiel MJ, Yilmaz OH, Iwashita T et al (2005) SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121:1109–1121
Kubota H, Avarbock MR, Brinster RL (2004) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci USA 101:16489–16494
Landgraf P, Rusu M, Sheridan R et al (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129:1401–1414
Li C, Heidt DG, Dalerba P et al (2007) Identification of pancreatic cancer stem cells. Cancer Res 67:1030–1037
Liao R, Sun J, Zhang L et al (2008) MicroRNAs play a role in the development of human hematopoietic stem cells. J Cell Biochem 104:805–817
Lu J, Guo S, Ebert BL et al (2008) MicroRNA-mediated control of cell fate in megakaryocyte-erythrocyte progenitors. Dev Cell 14:843–853
Majumdar MK, Keane-Moore M, Buyaner D et al (2003) Characterization and functionality of cell surface molecules on human mesenchymal stem cells. J Biomed Sci 10:228–241
Mallanna SK, Rizzino A (2010) Emerging roles of microRNAs in the control of embryonic stem cells and the generation of induced pluripotent stem cells. Dev Biol 344:16–25
Merkerova M, Vasikova A, Belickova M et al (2009) MicroRNA expression profiles in umbilical cord blood cell lineages. Stem Cells Dev 19(1):17–26
Merkle FT, Alvarez-Buylla A (2006) Neural stem cells in mammalian development. Curr Opin Cell Biol 18:704–709
Miyoshi N, Ishii H, Nagano H et al (2011) Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell 8:633–638
Mori T, Buffo A, Gotz M (2005) The novel roles of glial cells revisited: the contribution of radial glia and astrocytes to neurogenesis. Curr Top Dev Biol 69:67–99
Ng YY, van Kessel B, Lokhorst HM et al (2004) Gene-expression profiling of CD34+ cells from various hematopoietic stem-cell sources reveals functional differences in stem-cell activity. J Leukoc Biol 75:314–323
Niehage C, Steenblock C, Pursche T et al (2011) The cell surface proteome of human mesenchymal stromal cells. PLoS One 6:e20399

Nijnik A, Woodbine L, Marchetti C et al (2007) DNA repair is limiting for haematopoietic stem cells during ageing. Nature 447:686–690
Novershtern N, Subramanian A, Lawton LN et al (2011) Densely interconnected transcriptional circuits control cell states in human hematopoiesis. Cell 144:296–309
Nunomura K, Nagano K, Itagaki C et al (2005) Cell surface labeling and mass spectrometry reveal diversity of cell surface markers and signaling molecules expressed in undifferentiated mouse embryonic stem cells. Mol Cell Proteomics 4:1968–1976
O'Brien CA, Pollett A, Gallinger S et al (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445:106–110
O'Connell RM, Chaudhuri AA, Rao DS et al (2010) MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. Proc Natl Acad Sci USA 107:14235–14240
Ogawa M (2002) Changing phenotypes of hematopoietic stem cells. Exp Hematol 30:3–6
Oh H, Bradfute SB, Gallardo TD et al (2003) Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci USA 100:12313–12318
Ooi AG, Sahoo D, Adorno M et al (2010) MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. Proc Natl Acad Sci USA 107:21505–21510
Orkin SH, Zon LI (2008) Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132:631–644
Pang R, Law WL, Chu AC et al (2010) A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. Cell Stem Cell 6:603–615
Pastrana E, Cheng LC, Doetsch F (2009) Simultaneous prospective purification of adult subventricular zone neural stem cells and their progeny. Proc Natl Acad Sci USA 106:6387–6392
Pennartz S, Belvindrah R, Tomiuk S et al (2004) Purification of neuronal precursors from the adult mouse brain: comprehensive gene expression analysis provides new insights into the control of cell migration, differentiation, and homeostasis. Mol Cell Neurosci 25:692–706
Pennartz S, Reiss S, Biloune R et al (2009) Generation of single-cell suspensions from mouse neural tissue. J Vis Exp 2009 Jul 7, 29(2):1267. doi:10.3791/1267
Petriv OI, Kuchenbauer F, Delaney AD et al (2010) Comprehensive microRNA expression profiling of the hematopoietic hierarchy. Proc Natl Acad Sci USA 107:15443–15448
Pfister O, Mouquet F, Jain M et al (2005) CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. Circ Res 97:52–61
Piccirillo SG, Vescovi AL (2006) Bone morphogenetic proteins regulate tumorigenicity in human glioblastoma stem cells. Ernst Schering Found Symp Proc 5:59–81
Pontier SM, Muller WJ (2009) Integrins in mammary-stem-cell biology and breast-cancer progression–a role in cancer stem cells? J Cell Sci 122:207–214
Quirici N, Soligo D, Bossolasco P et al (2002) Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp Hematol 30:783–791
Rafii S, Lyden D (2003) Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 9:702–712
Ramalho-Santos M, Yoon S, Matsuzaki Y et al (2002) "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 298:597–600
Ricci-Vitiani L, Lombardi DG, Pilozzi E et al (2007) Identification and expansion of human colon-cancer-initiating cells. Nature 445:111–115
Rossi DJ, Bryder D, Zahn JM et al (2005) Cell intrinsic alterations underlie hematopoietic stem cell aging. Proc Natl Acad Sci USA 102:9194–9199
Schiedlmeier B, Santos AC, Ribeiro A et al (2007) HOXB4's road map to stem cell expansion. Proc Natl Acad Sci USA 104:16952–16957
Schmelzer E, Reid LM (2008) EpCAM expression in normal, non-pathological tissues. Front Biosci 13:3096–3100
Schmelzer E, Zhang L, Bruce A et al (2007) Human hepatic stem cells from fetal and postnatal donors. J Exp Med 204:1973–1987
Seandel M, James D, Shmelkov SV et al (2007) Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature 449:346–350

Seidenfaden R, Desoeuvre A, Bosio A et al (2006) Glial conversion of SVZ-derived committed neuronal precursors after ectopic grafting into the adult brain. Mol Cell Neurosci 32:187–198
Shackleton M, Vaillant F, Simpson KJ et al (2006) Generation of a functional mammary gland from a single stem cell. Nature 439:84–88
Shi S, Gronthos S (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 18:696–704
Shimono Y, Zabala M, Cho RW et al (2009) Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell 138:592–603
Singh SK, Clarke ID, Terasaki M et al (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res 63:5821–5828
Singh SK, Clarke ID, Hide T et al (2004a) Cancer stem cells in nervous system tumors. Oncogene 23:7267–7273
Singh SK, Hawkins C, Clarke ID et al (2004b) Identification of human brain tumour initiating cells. Nature 432:396–401
Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marbán E (2007) Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 115(7):896–908
Son MJ, Woolard K, Nam DH et al (2009) SSEA-1 is an enrichment marker for tumor-initiating cells in human glioblastoma. Cell Stem Cell 4:440–452
Steidl U, Kronenwett R, Rohr UP et al (2002) Gene expression profiling identifies significant differences between the molecular phenotypes of bone marrow-derived and circulating human CD34+ hematopoietic stem cells. Blood 99:2037–2044
Stingl J, Eirew P, Ricketson I et al (2006) Purification and unique properties of mammary epithelial stem cells. Nature 439:993–997
Storms R, Trujillo A, Springer J (1999) Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc Natl Acad Sci USA 96:9118–9123
Sturzu AC, Wu SM (2011) Developmental and regenerative biology of multipotent cardiovascular progenitor cells. Circ Res 108:353–364
Suh MR, Lee Y, Kim JY et al (2004) Human embryonic stem cells express a unique set of microRNAs. Dev Biol 270:488–498
Sun S, Guo Z, Xiao X et al (2003) Isolation of mouse marrow mesenchymal progenitors by a novel and reliable method. Stem Cells 21:527–535
Tang C, Ang BT, Pervaiz S (2007) Cancer stem cell: target for anti-cancer therapy. FASEB J 21:3777–3785
Timmermans F, Plum J, Yoder MC et al (2009) Endothelial progenitor cells: identity defined? J Cell Mol Med 13:87–102
Tondreau T, Meuleman N, Delforge A et al (2005) Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. Stem Cells 23:1105–1112
Toren A, Bielorai B, Jacob-Hirsch J et al (2005) CD133-positive hematopoietic stem cell "stemness" genes contain many genes mutated or abnormally expressed in leukemia. Stem Cells 23:1142–1153
Wagner W, Ansorge A, Wirkner U et al (2004) Molecular evidence for stem cell function of the slow-dividing fraction among human hematopoietic progenitor cells by genome-wide analysis. Blood 104:675–686
Wilson KD, Venkatasubrahmanyam S, Jia F et al (2009) MicroRNA profiling of human-induced pluripotent stem cells. Stem Cells Dev 18:749–758
Wollscheid B, Bausch-Fluck D, Henderson C et al (2009) Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. Nat Biotechnol 27:378–386
Xiao C, Calado DP, Galler G et al (2007) MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell 131:146–159
Yang ZF, Ho DW, Ng MN et al (2008) Significance of CD90+ cancer stem cells in human liver cancer. Cancer Cell 13(2):153–166

Yovchev MI, Grozdanov PN, Joseph B et al (2007) Novel hepatic progenitor cell surface markers in the adult rat liver. Hepatology 45:139–149

Yovchev MI, Grozdanov PN, Zhou H et al (2008) Identification of adult hepatic progenitor cells capable of repopulating injured rat liver. Hepatology 47:636–647

Zhan M, Miller CP, Papayannopoulou T et al (2007) MicroRNA expression dynamics during murine and human erythroid differentiation. Exp Hematol 35:1015–1025

Zhou B, Wang S, Mayr C et al (2007) miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. Proc Natl Acad Sci USA 104:7080–7085

Chapter 7
Human Embryonic Stem Cells

Mikael C.O. Englund, Peter Sartipy, and Johan Hyllner

Abstract Stem cells can be isolated from a variety of sources and they are typically classified based on their tissue of origin. Embryonic stem cells are, as the name indicates, derived from the inner cell mass of pre-implantation stage blastocysts at day 5–7 post fertilisation. These cells possess qualities such as pluripotency and a seemingly limitless capacity to proliferate *in vitro* in their undifferentiated state. Embryonic stem cells were first derived from mouse embryos in the early 1980s but have now been derived from a number of different species including rat, rabbit, sheep, horse and human. This chapter focuses on human embryonic stem cells and describes techniques used for their derivation and culture. In addition, the basic properties of these cells are illustrated, including some examples of their capacity to differentiate to various precursors and functional cell types. Finally, some areas of applications for these cells are discussed with emphasis on their possible future use in regenerative medicine.

7.1 Introduction

The developments in the human pluripotent stem cells field during the last decade are remarkable, and the scientific achievements made have substantially furthered our understanding of the opportunities that these cells provide for basic and applied research as well as for future regenerative medicine applications. There has been rapid progress in the development of improved derivation and culture technologies for human embryonic stem (hES) cells since the initial derivation of stable cell lines

M.C.O. Englund, Ph.D. (✉) • P. Sartipy, Ph.D.
Cellectis stem cells, Arvid Wallgrens Backe 20, SE-413 46 Göteborg, Sweden
e-mail: mikael.englund@cellectis.com

J. Hyllner, Ph.D.
Cellectis stem cells, Arvid Wallgrens Backe 20, SE-413 46 Göteborg, Sweden

Department of Physics, Chemistry and Biology, Linköping University,
SE-581 83 Linköping, Sweden

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_7,

in 1998 (Thomson et al. 1998). One of the main driving forces behind this is the aim to generate high quality, clinically compliant, hES cell lines which can be used for future cell therapy in humans. As such, the cell lines need to be manufactured according to good manufacturing practice (GMP) in order to comply with good clinical practice (GCP) which is a set of internationally recognised ethical and scientific quality requirements that must be observed for designing, conducting, recording and reporting clinical trials that involve the participation of human subjects (2001/20/EC 2001; 2005/28/EC 2005). In addition, there are more immediate opportunities to use hES cells and their derivatives as *in vitro* tools to study for example human development and genetic diseases. Furthermore, the cells are also expected to contribute to improvements of the models currently used in drug discovery and by providing a source of a variety of human specialised cells which then can be manufactured under standardised conditions. However, in order to realise the opportunities that human pluripotent stem cells provide, there are a number of challenges that need to be addressed. Cost-efficient culture conditions which allow large scale production of the undifferentiated cells is one, efficient and robust protocols for the process of differentiation of the pluripotent cells to the desired end point is another. Characterisation and quality control also require specific attention and monitoring the phenotype and genomic stability of the cells during expansion and propagation is critical. For any kind of therapeutic applications, regulatory compliance needs to be factored in as well. Ideally culture systems would be based around fully defined components, using small molecules with a decreased dependency of biologics. There is a lot of effort spent on genetically modifying the cells, e.g. to make cells overexpress genes linked to desirable functionality such as metabolising enzymes and also to generate reporter lines. With the last years' advancements in the generation of induced pluripotent stem (iPS) cells, the technologies used for genetically modifying cells has certainly fuelled further research in this area of engineering stem cells. Further details on the iPS cells and the opportunities and challenges they provide are reviewed in a separate chapter of this book and will not be discussed further here. Below, we will cover basic aspects on the derivation process of hES cells and highlight different culture conditions for maintenance of the undifferentiated cells. We will also describe various ways to characterise hES cells in order to verify their unique properties. The pluripotency of the cells will be illustrated with some examples of differentiated cell types which can be generated from the hES cells, and applications in regenerative medicine will be discussed.

7.2 Derivation/Classification

7.2.1 Derivation

The different sources of human embryos which have been successfully used for derivation of new hES cell lines are blastocysts (Thomson et al. 1998), morulae

(Strelchenko et al. 2004), late-arrested embryo (Feki et al. 2008; Gavrilov et al. 2009; Zhang et al. 2006) or blastomere (Geens et al. 2009; Klimanskaya et al. 2006).

Initially, the method was adapted from the previously developed protocol for mouse ES cells (Evans and Kaufman 1981; Martin 1981). The substantial species differences and the lack of appropriate culture medium for human embryos were probably part of reason why it was not until the late 1990s the first human embryonic stem cell line was isolated. Discrepancies in intracellular pathway signalling between mice and man have been demonstrated as one explanation to the differences in culture requirements (Brandenberger et al. 2004; Rho et al. 2006; Xu et al. 2002). In 1994, Bongso and co-workers managed to isolate and to some extent propagate inner cell masses (ICMs) from human blastocysts and these cells displayed stem-cell like properties (Bongso et al. 1994). This achievement in concert with the successful derivation of non-human primate ES cell lines in the mid 1990s (Thomson et al. 1995, 1996) paved the way for the subsequent derivation of stable hES cell lines in 1998 (Thomson et al. 1998). After this, the generation of a large number of hES cell lines have been reported, exceeding well over 1,000 different lines (Loser et al. 2010).

The majority of hES cell lines derived thus far have utilised donated surplus embryos from assisted conception laboratories, i.e. from the procedure of *in vitro* fertilisation (IVF). Both fresh and previously frozen material can be used for stem cell derivation. The embryos are cultured to the blastocyst stage before measures are taken to isolate the ICM cells. In order to accomplish this, the expanded blastocyst is initially treated with pronase to digest the surrounding zona pellucida. Alternatives to the use of pronase for removing the zona are Tyrode's acid solution or mechanical opening, which circumvents the use of xeno-derived pronase. Spontaneously hatched blastocysts can also be processed further without the need for zona pellucida removal (Heins et al. 2004). Subsequently, the blastocyst is treated with mouse antibodies directed against human trophoectoderm cells and guinea pig complement components. This process, called immunosurgery, lyses the cells by an antibody/complement reaction leaving the inner cell mass cell mostly intact, and these cells can then be sub-cultured further on a layer of mitotically inactivated mouse embryonic fibroblasts feeder cells (mEF cells). The initial outgrowth from the inner cell mass cells is usually dissected mechanically under the microscope and transferred to new culture dishes after 1–2 weeks. An established hES cell line is typically passaged every 5–10 days, depending on culture method and population doubling time. As soon as the hES cells are growing in a way allowing culture expansion, low passage samples should be cryopreserved as a seed bank, and also as larger well characterised Master Cell Banks (MCB). From these MCBs, Working Cell Banks (WCB) can be established, for further expansion and various endpoint applications. If the aim is to derive hES cells for downstream clinical use, i.e. to generate therapeutic cells, then the whole procedure needs to comply with regulatory demands, such as current Good Manufacturing Practice (cGMP), but also comply with ethical regulations (Crook et al. 2007; Murdoch et al. 2012).

The methodology for ICM isolation briefly described here is depending on careful monitoring and execution for successful results since the viability of the resulting ICM isolate is fragile. A success rate in hES cell line generation of at least

5–10 % is reasonable to expect and up to more than 30 % success rate has been reported (Chen et al. 2009; Sjogren et al. 2004). Negative results could be related to sub optimal culture conditions or other technical issues. Blastocyst quality is also an influencing factor for successful derivation of hES cell lines, and the use of higher quality embryos leads to a greater success rate (Lerou et al. 2008b). However hES cell lines have been established from blastocysts graded as of low quality (Dokras et al. 1993; Heins et al. 2004; Lerou et al. 2008a) and even from non-viable embryos, albeit with questionable quality (Feki et al. 2008). Assuming embryo culture is well conducted it appears that derivation from previously cryopreserved embryos or blastocysts should be as successful as derivation from fresh material (Sjogren et al. 2004). Far from all cryopreserved embryos are eligible and donated for research activities. However, there seems to be a positive correlation between the level of information and support from the IVF clinic staff and the treated patient's willingness to donate surplus material for research (Brett et al. 2009).

7.2.2 Classification

The essence of a hES cell is its ability to differentiate into other, more specialised cell types, such as muscle cells, neurons, connective tissue and epithelial cells, to mention a few. The hES cells are pluripotent, i.e. they can form cells representing the three germ layers endoderm, ectoderm, and mesoderm. To assess the quality of a recently derived hES cell line, the expression or selective absence of various surface markers, transcription factors, and other properties of the assumed pluripotent hES cells are monitored. Several of these markers have been found closely associated to the pluripotent state of hES cells and researchers normally apply a panel of several markers for the characterisation procedure (Heins et al. 2004; Thomson et al. 1998). Briefly, they include

- Cell membrane bound surface markers like the glycol lipids SSEA-1,-3, -4; and the keratin sulphate molecules TRA-1-60, -1-81.
- Transcription factors such as Oct-4, Sox2, and Nanog.
- Telomerase activity, as a measure of the hES cells ability to continuously go through mitosis.
- Alkaline phosphatase activity.
- *In vitro* pluripotency, assayed for by using markers for endo-, ecto-, and mesoderm on spontaneously differentiated cell material. Commonly used markers are the transcription factor forkhead box A2 (Foxa2) transcription factor for endoderm, β-III-tubulin for ectoderm and Arterial Smooth Muscle Actin (ASMA) as a marker for Mesoderm.
- *In vivo* pluripotency, assayed for by xenografting hES cells into an immunodeficient mouse and consequently analysing the resulting teratoma for endo-, ecto-, and mesoderm derivatives. Typically, the cells are placed under the kidney capsule but other areas have also been used such as testis and skeletal muscle.

In Fig. 7.1a, the nuclei of a confluent layer of hES cells have been stained with an antibody for the transcription factor Nanog to illustrate its presence. Nanog was

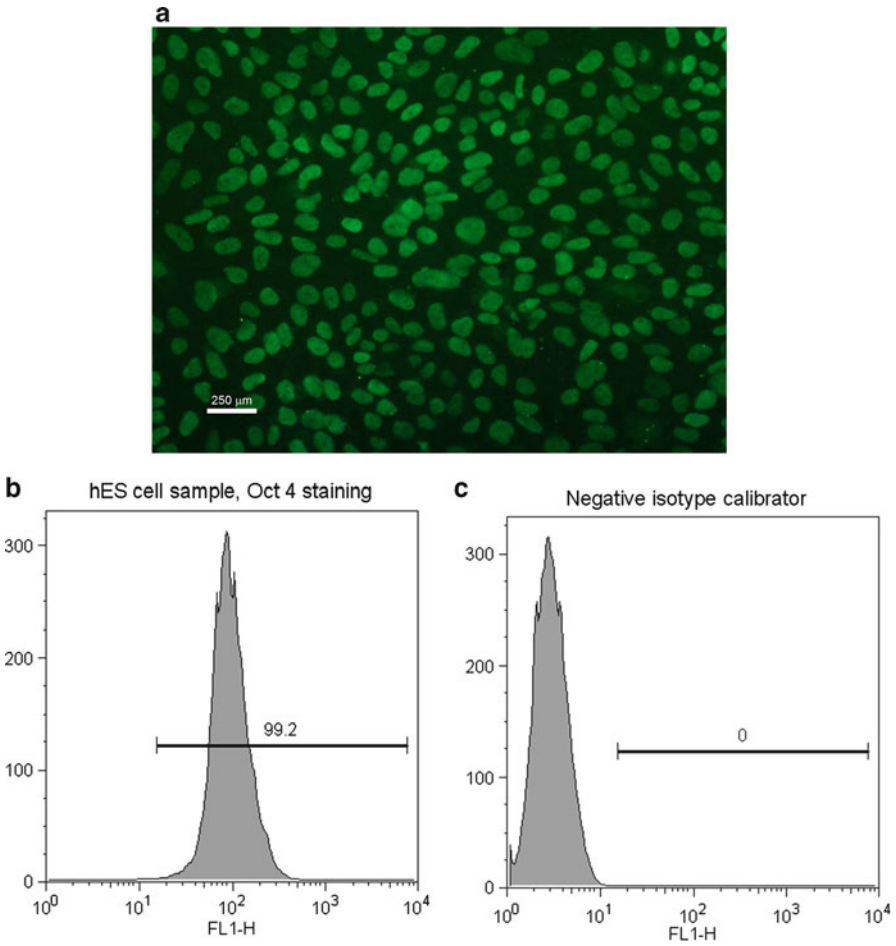


Fig. 7.1 (**a**) Nanog nuclear staining of a confluent layer of hES cells. This methodology, known as immunocytochemistry, is commonly used to illustrate the presence of various factors and markers linked to certain properties of cells, e.g. pluripotency of hES cells. (**b**) Flow Cytometry (*FC*) diagram of hES cells positive to 99.2 % for Oct-4 transcription factor. By using quantitative methods like FC a more exact determination of the characteristics of stem cells is possible, compared to qualitative methods. (**c**) FC isotype negative control. This illustrates how cells negative for the marker appear in the FC analysis

simultaneously discovered in 2003 as a pluripotency sustaining factor by Chambers and Mitsui (Chambers et al. 2003; Mitsui et al. 2003). Flow Cytometry (FC) allows a more exact quantification of markers than microscopic observations of immunostainings, and several markers could be analysed simultaneously, e.g. to asses co- expression of more than marker. In Fig. 7.1b, an FC graph illustrates a population of Oct-4 positive cells as an indication of pluripotency, with Fig. 7.1c illustrating the isotype negative control for the assay as a comparison . In addition to the above examples of properties linked to pluripotency, the genomic stability of the hES cells is normally assessed,

employing standard G-banding karyotyping or more high resolution techniques such as high density array based SNP analysis. The absence of pathogens is also normally confirmed as well as the thawing recovery rate after cryo-preservation.

The International Stem Cell Forum (ISCF) launched a characterisation initiative few years ago (The International Stem Cell Initiative or ISCI) as a global collaborative effort to conclude and harmonise basic methodology and criteria for the derivation, characterisation and maintenances of hES cells (Adewumi et al. 2007), also discussed by Stephenson et al. (2007). The consensus of the scientific community in concert with the regulatory bodies for the major markets will be central to the eventual development of medical applications of hES cells.

7.3 Culture Conditions

7.3.1 General

It is anticipated that hES cells are more sensitive to sub optimal culture conditions than common somatic cell lines, hence demanding a more stable and controlled environment and a precise culture medium formulation to maintain the undifferentiated and pluripotent state during long term culture. A number of critical parameters should be accounted for when setting up a hES cell culture laboratory, including:

- Laboratory facilities-there should be a dedicated fit for purpose built and secluded cell culture area with
  - Clean filtered air and stable temperature, preferable positive pressure.
  - A minimum of consumables storage in the direct cell culture area.
  - Good cleaning routines and easily cleaned equipment and surfaces.
  - High quality equipment that is regularly serviced and calibrated where appropriate, cell culture incubators maintaining a stable climate to avoid changes in pH, temperature and osmolality of the culture medium.
  - Heated stages fitted to the microscopes to avoid a decrease in culture temperature when outside of the incubator for inspection or manipulation.
- Aseptic handling-especially important if cultures are not supplemented with antibiotics. The staff should be trained and audited accordingly.
- Contact materials-all materials should preferable be tested for embryotoxicity or be of IVF grade. Surface cleaning detergents should be non abrasive, non volatile and non toxic.
- High quality culture reagents, such as medium, growth factors and other reagents and solutions.
- Good cell culture laboratory routines in general, regular testing for mycoplasma, quarantine routines and preferable a quality system in place that regulates version control of protocols and non conformances.

7.3.2 Culture Propagation Techniques-Cut and Paste

The classic method for culture of hES cells is in co-cultures with supporting feeder cells, typically mitotically inactivated mouse embryonic fibroblasts, that provide support in terms of conditioning of the culture medium, surface matrix components and other direct cell-cell interactions (Ellerstrom et al. 2007; Heins et al. 2004; Thomson et al. 1998). Critical parameters for high quality hES cell colonies are the quality of the feeder cells, as well as their density and capacity to condition the culture medium. As further discussed below, other hES cell culture systems free of a feeder layer per se could still rely on medium being conditioned by feeder cells, thus the medium contain factors favourable for maintaining the hES cells in a pluripotent state (Prowse et al. 2005; Lim and Bodnar 2002), The hES cell culture medium could also be totally free of feeder cell influence and have more or less defined components as discussed below. The original method for propagation of hES cell cultures on feeder cells is by mechanical dissection. A fine sharp object, like a capillary drawn out over a flame, micro scalpels or other bespoke stem cell cutting tools are used for slicing up a mature colony of hES cells into smaller pieces that is subsequently transferred to a new culture vessel with feeder cells. In Fig. 7.2, a hES cell colony has been sectioned and some pieces removed to illustrate the technique. hES Cell colonies typically grow to rounded flat and homogenous

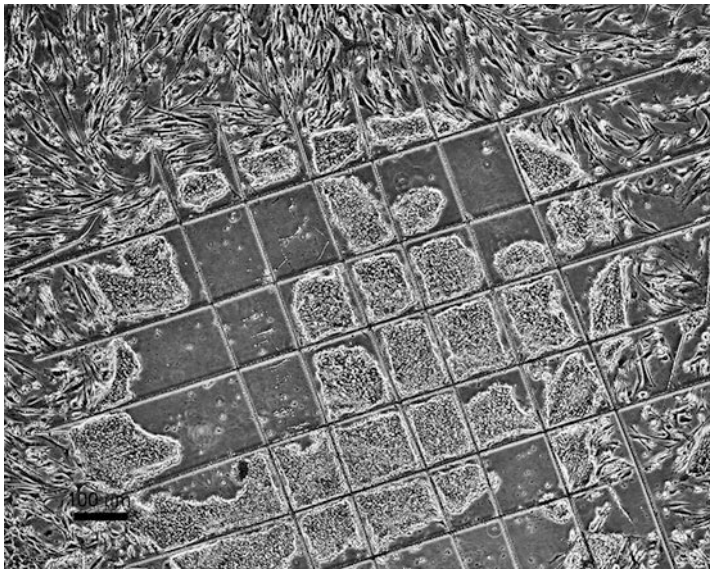


Fig. 7.2 Manually dissected hES cell colony, demonstrating the principles of this propagation method. The mature hES cell colony is sliced up in small fragments using a fine sharp object like a pulled-out glass capillary or a micro scalpel. The colony fragments are subsequently detached from the culture dish and transferred to a new dish with a fresh feeder cell layer. This procedure is typically repeated every 5–7 days, and culture medium is refreshed in between

colonies, a layer just a single, or a few cells thick. The colonies are clearly visible by eye and can be sizeable, in the millimetre scale. The culture schedules for individual hES cell line often have to be titrated, however, the colonies generally need to be passaged every 5–7 days. If not, the hES cell colony will eventually start to show signs of differentiation, with random 3D structures appearing, migration of fibroblast-like cells and even formation of spontaneously beating cells and neural-likeoutgrowths. In addition, the feeder layer will deteriorate as well. Typically, the culture vessels with the feeder layer are prepared some time in advance, allowing conditioning of the culture medium. During the course of culture, a fraction or the whole of the culture medium is normally replaced at intervals. Centre well dishes like the ones used for IVF are commonly used since they are designed to allow manipulation of their content, have a rim compartment that should contain culture medium or sterile buffered solution in order to minimise changes in osmolality of the centre well, and not the least, they are subjected to a rigorous quality control. Nevertheless, a number of different culture vessels can be used. When simultaneously maintaining more than one hES cell line in culture, routines should be established so the risk of cross contamination between lines is eliminated.

7.3.3 Enzyme Mediated Passage

The cut and paste method for hES cell passage described above is very labour intensive and requires staff skilled in micro dissection. It is also practically impossible to acquire enough cells for large scale experiments, not to mention sufficient number of cells for use in compound screening or future regenerative medicine applications. As a consequence, protocols for enzymatic digestion of hES cell colonies have been developed and reported by several groups and there are several commercially available culture systems allowing feeder cell independent stem cell culture in confluent monolayers with enzyme mediated passage, with more or less defined components. The important aspect of these culture system is their ability to support hES cell growth on either a biologic or synthetic matrix without involvement of feeder cells and the unknown parameters that would add. The advantages are apparent; it is less time consuming to passage the hES cells and a larger number of cells can be cultured by a single person. After establishment and initial mechanical passage, the hES cells have been made progressively tolerant to enzymatic digestion, eventually allowing the cell colonies to be dissociated from old culture vessels and transferred into new ones with maintained viability. The cell colonies can be dissociated by enzymes, such as trypsin and collagenase IV, and also by treatment with EDTA. Needless to say, a system based on enzymatic digestion and large scale cultures need to be robust and validated to support long term pluripotency. Such a system is also ideal for automation of stem cell cultures. What is lost by simultaneously digesting all hES cell colonies in a culture vessel is the high level of control that the mechanical approach offers, i.e. the selection of morphologically perfect specimens over differentiated, or partly differentiated cells. Therefore, it is not unlikely that, to

some extent, the cut and paste method will be kept on the back burner in many laboratories as a mean to go back to selective high quality cultures as starting material for transfer to other culture systems.

When hES cell colonies or confluent layers are enzymatically digested, two principles of passage can be applied. Either the cells can be dissociated to clusters of a few hundred cells or to a single cell suspension. Cell viability is largely preserved in the former procedure, however, the stress inflicted on the hES cells in the latter will lead to apoptosis (Watanabe et al. 2007). Watanabe and co-workers made a significant finding of a rho-kinase inhibitor that prevents apoptosis, thus allowing single cell suspensions of hES cell to maintain viability. The substance also improved cell survival after cryopreservation (Li et al. 2009). This means that it is feasible to propagate hES cells in a highly reproducible manner and importantly, to seed them into culture vessels at exact numbers, which is a prerequisite for e.g. compound screening campaigns or other applications that require absolute control over seeding densities. Processing hES cells in quantified single cell suspensions also realises the possibility to, by automated means, propagate the cells in a programmed way, with exact concentrations of cells seeded as desired. Figure 7.3 illustrates a confluent layer of hES cells in a feeder-free culture system.

Concerns were raised in 2004 by Draper and co-workers, demonstrating that prolonged exposure of hES cell cultures to enzymes would cause chromosomal aberrations (Draper et al. 2004), however other studies have later demonstrated that hES cells can be enzymatically propagated over longer periods of time with stable characteristics (Sjogren-Jansson et al. 2005; Suemori et al. 2006). As Catalina and colleagues suggest, the individual hES cell lines may have different pre dispositions

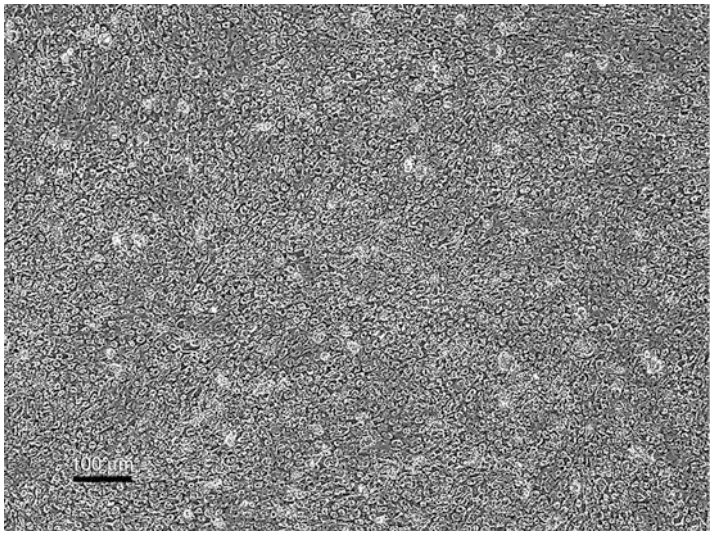


Fig. 7.3 A confluent monolayer of hES cells cultured without the presence of feeder cells. This technique allows scaled up, and also automated hES cell culture

to chromosomal instability as well (Catalina et al. 2008). To avoid suspicions of chromosomal changes, all hES cell cultures should either be regularly karyotyped or only cultured in a passage window that is unlikely to allow chromosomal deviations to appear and propagate. hES cells not regularly subjected to enzymatic digestion, such as cut and paste cultures, have been shown to sustain a stable karyotype for up to almost 2 years in continuous culture (Caisander et al. 2006).

7.4 Scaling Up Cultures

For any clinical or industrial application of either undifferentiated hES cells or derivatives thereof, a scaled up production is required, possibly automated as well. Protocols for directed differentiation of naïve hES cells to a specialised cell fate often includes selection in some way, hence the cell population will be decimated. It has been estimated that for a hES cell based repair of a heart damaged by ischemia, hES cells in the range of billions would be required (Passier and Mummery 2005). Clearly, the volume of the starting material is depending on the procedure of differentiation the cells to the desired end point and it is very difficult to today predict the demands for starting material for a future cell-based therapy . Efficacy is one challenge as well as efficiency, the cost of the process and purity of cell populations are two examples. To achieve billions of stem cells as a starting point is not realistic to achieve with the old traditional ways of hES cell culture, i.e. manual dissection in single culture dishes, so the need for massive amounts of starting material has been a clear driver for the development of scalable culture systems, such as these discussed in the previous section. The scaled up culture process for hES cells need to be as robust and simplistic as possible, to generate the necessary reproducibility and to be cost effective. Thus, the number of unknowns in the process should be kept to a minimum and ideally a hES cell culture system should be feeder independent and based on defined media and matrix, synthetic or recombinant. The culture procedure must also be subjected to quality control, and preferable GLP procedures should apply. The application of MCBs and WCBs will contribute to reproducibility and robustness in the culture system.

Recent progress have been reported where hES cells successfully have been cultured in amounts required for conducting compound screening campaigns in multiwell plate formats (Desbordes et al. 2008; Thomas et al. 2009; Andrews et al. 2010). These achievements are essential for the use of hES cells in for example drug discovery.

The definition and development of industrial cell production standards needs to be addressed, since scaled up hES cell culture is still a relatively new technology,. the ability to manipulate hES cell cultures as single cell suspensions is a first critical step towards reproducible scaled up culture, and not the least, distribution of even cell numbers in multiwell plates for screening. The possibility of adding wholly or partly automated cell culture technology will further strengthen the capability to supply a consistent quality of cells (Thomas et al. 2009). Future widespread use of hES cell derivatives in bio reactors will also demand cell numbers of an industrial scale.

7.5 Deriving Xeno Free hES Cells and hES Cells for Clinical Use

Routinely, all mammalian cell culture rely heavily on reagents sources from animals, e.g. serum, amino acids, albumin, various matrices like collagen and other factors. This is cost effective and gives enough consistency for the majority of applications. On the other hand, the undefined culture condition that is a consequence of the common use of bovine serum as a source of growth factors may be of concern for different reasons. One apprehension is from the aspect of reproducibility and possible batch-to-batch variation. Maybe of more interest in the context of the stem cell therapy area, is the risk of contamination by prions, viruses or other zoonoses of the cells cultured in contact with the animal material. Concerns were raised when it was observed that hES cells cultured in contact with animal material incorporated and expressed animal sialic acid, however, this has also been demonstrated to be reversible (Heiskanen et al. 2007; Martin et al. 2005; Nasonkin and Koliatsos 2006). To address this potential problem, derivation, propagation and banking of hES cell lines strictly without any contact with animal-sourced material, i.e. under "xeno-free" conditions, have been reported by several groups (Ellerstrom et al. 2006; Ludwig et al. 2006; Rajala et al. 2007; Richards et al. 2004; Ilic et al. 2012).

This is still substantially more expensive than regular hES cell culture due to the high costs of the reagents, but also the need for hardware as well as routines separated from the non-xeno free cell cultures. The International Stem Cell Banking Initiative is a group of stake holders striving to harmonise guidelines for stem cell banking worldwide, with the goal of delivering the best practice for clinical grade stem cell delivery (Crook et al. 2010).

One of the great hopes of hES cells are to utilise them as raw materials for tissue engineering and replacement for damaged organs or tissues. A number of clinical situations have been addressed as potential targets for this kind of approach. A therapy where insulin-dependent type-1 diabetics could receive functional beta cells that would integrate and normalise blood glucose levels would revolutionise thousands of lives of those affected by this auto immune disease that targets and destroys the endogenous insulin producing cells. Replacing damaged neurons in patients suffering from spinal cord injuries could mean the difference between confinement to a wheel chair and normal mobility. The examples are plenty and great hopes are put to this future development by many patient groups and clinicians.

Since the above described scenarios include the transplantation of living cells into patients, the regulatory framework is elaborate. For any future cell-based therapy, Good Clinical Practice (GCP) is a requirement for clinical trials, and this includes that the cells have been sourced and produced according to cGMP. This is a regulatory framework ensuring that an end product meets pre-set specifications. For a putative therapy based on hES cell derived functional cells, it includes the regulation of processes for

- Donation and procurement of the starting materials, e.g. blastocysts and feeder cells
- Testing and quality control
- Processing and manufacturing of the undifferentiated cells as well as all the procedure of generation differentiated cells.
- Traceability of all reagents and materials as well as release criteria.

In 2007, Crook and co-workers published the first six hES cell lines derived and further processed in line with GMP regulations (Crook et al. 2007) and the most recent efforts in this area was published in 2012 (Ilic et al. 2012). These are an important steps towards the clinic; however, there are still a big challenges to overcome; to develop differentiated functional cells, verified and validated, of clinical value. Also, the six GMP compliant hES cell lines derived by Crook and co workers were not derived in absence of animal material, i.e., they cannot be defined as xeno-free. However, from a GMP perspective, the use of reagents sourced from animals, like serum, is acceptable, although it has been argued that clinical grade hES cells, or hES cell derived cells need to be derived xeno-free (Unger et al. 2008). Nevertheless, in 2009, Geron Corporation was granted permission from the US Food and Drug Administration (FDA) to initiate the first phase 1 clinical trials for a spinal cord injury therapy, based on specialised cells derived from the hES cell line H1 (Alper 2009; Barde 2009). This hES cell line was originally cultured in contact with animal components and without all the intricate documentation and assurance that GMP compliance gives (Geron 2009; Thomson et al. 1998). As of late 2011, the Geron clinical trials have been stopped. As this chapters is written, the company Advanced Cell Technology are pursuing clinical trials for two conditions affecting the eye; Stargardt's macular dystrophy and dry age-related macular degeneration (http://www.advancedcell.com/patients/clinical-trial-information/).

In order to minimise the dependency on undefined contributions from feeder cells, serum and complex culture matrices like Matrigel (BD Biosciences), efforts have been made to map the hES cell culture requirements for defined culture systems, resulting in commercially available defined culture systems for hES cells and iPS cells. For clinical use of hES cell or hES cell derived cells, cultures free of any animal components would desirably decrease the number of unknowns in the equation as would replacing biologics with small molecules. In addition to the biological and regulatory challenges, the industry and institutions need to generate enough cells suitable for therapeutic use and at the same time make it financially sound.

7.6 Differentiation Capacity and Their Precursors

The ability of hES cells to differentiate into virtually any specialised cell type present in the adult body is one of the key features of these cells. Spontaneous differentiation occurs *in vitro* when the cells are cultured in conditions lacking the appropriate components that sustain pluripotency. In addition, several protocols for

directed differentiation of hES cells into various specialised cell types have been reported in which the cells are guided along lineage restricted pathways to generate relatively pure populations of cells. A detailed review on this topic is beyond the scope of the present chapter and here we only exemplify the differentiation capacity of hES cells using two brief examples; cardiomyocytes and hepatocytes.

The differentiation of hES cells towards the cardiac lineage can be observed through the appearance of clusters of spontaneously contracting cells, as originally reported in 2000 (Itskovitz-Eldor et al. 2000). Different approaches have been developed to induce hES cells to differentiate to cardiomyocytes in culture. One is based on the formation of embryoid body-like structures under the influence of various cocktails of growth factors or small molecules in attempts to recapitulate heart development *in vivo* (Kehat et al. 2001; Yang et al. 2008). Another method is to utilise a co-culture system with hES cells and END-2 cells (a visceral endoderm mouse cell line), in which the pluripotent stem cells are directly exposed to cell-cell interactions as well as the secretome of the END-2 cells (Mummery et al. 2003). More recently, deriving cardiomyocytes from monolayers of hES and iPS cells have been demonstrated (Laflamme et al. 2007; Uosaki et al. 2011) and from hES cells cultured under defined conditions using a directed differentiation approach using small molecules (Parsons et al. 2011). Molecular, pharmacological, and electrophysiological studies have characterised hES cell-derived cardiomyocytes to various extents and these cells express many cardiac markers, including transcription factors, structural proteins, ion-channels, and different junction proteins (Beqqali et al. 2006; Synnergren et al. 2008). Despite the similarities with their adult counterparts, hES cell-derived cardiomyocytes still seem to mainly display a foetal cardiac phenotype, and future research is needed to develop protocols which can sustain *in vitro* maturation of the cells towards a phenotype more close to the adult human cardiomyocyte. For drug discovery applications it would be desirable to generate pure populations of e.g. ventricular cardiomyocytes, to study specific targets.

In 2003, the first report appeared which described the generation of hepatic-lineage cells from hES cells, with several publications following (Agarwal et al. 2008; Baharvand et al. 2008; Cai et al. 2007; Rambhatla et al. 2003) and more recently by Medine and co-workers (Medine et al. 2011). It is however a major impediment to generate truly metabolically competent cells, i.e., cells expressing relevant enzymatic activities. No studies to date have shown activity levels of different Cytochrome P450 enzymes that resembles those of freshly isolated human primary hepatocytes. Rather, the accumulated published work implicates that obtaining fully functional cells from hES cells is a major challenge (D'Amour et al. 2006; Guguen-Guillouzo et al. 2010).

7.7 Potential Applications for Therapies

Therapeutic applications based on hES cells add several dimensions to the challenges regarding hES cells culture, differentiation, and purification as discussed above. In addition, critical aspects such as safety and efficacy needs to be clarified

in detail before such cells can progress to clinical trials. Indeed, much advancement has been made towards these ends and there are many diseases that are envisioned as suitable for targeting with stem cell therapies. For example, despite important advances in pharmacological therapies and organ transplantation, heart failure represents an enormous clinical problem. The limitations of the current interventions have driven the search for stem cell-based techniques to repair and regenerate heart muscle. The opportunity to create the major cell types present in the human heart (i.e., cardiomyocytes, smooth muscle cells, and endothelial cells) from pluripotent stem cells and subsequently transplanting these into the site of injury is an appealing strategy. Pre-clinical studies have begun to investigate hES cell-based heart regeneration, and initial studies provided positive encouragement and demonstrated short term (4 weeks) functional improvement following transplantation of hES cell-derived cardiomyocytes to injured myocardium (Caspi et al. 2007; Laflamme et al. 2007; Leor et al. 2007). However, it was later demonstrated that the functional improvement was transient and no differences were observed between the cardiomyocyte transplanted group and the control animals at 12 weeks post-myocardial infarction (van Laake et al. 2007). Also, formation of fibrous tissue around the graft has been observed, hampering electrophysiological integration. However when hES cell derived cardiomyocytes were co-transplanted with hES cell derived endothelial cells and/or cardiac progenitor cells into mouse hearts, functional capillaries were formed and the cardiomyocytes were found to survive for up to 24 weeks. The formation of capillaries suggests the possibility of an increased blood supply to the graft area. Although the understanding of the mechanism of action is limited at present, it appears that transplantation of cells to the injured heart has some beneficial effects but there are other possibly prohibitive mechanisms that need to be elucidated further (van Laake et al. 2009, 2010).

The possibility of generating relevant numbers and quality of hepatocytes for bio-artificial liver support technology and possibly also for *in vivo* liver regeneration is a thrilling prospective (Dalgetty et al. 2009; Medine et al. 2011). In addition, getting an unlimited access to competent liver cells would be of exceptional use for studying drug targets, metabolism and toxicity (Jensen et al. 2009). On the other hand, *in vitro* differentiation of hES cells towards the hepatic lineage clearly is a challenging task (Snykers et al. 2009). *In vivo*, the insulin producing beta cells are partly developed along the same pathway as liver cells, and these cells are also of great interest as a potential cure for insulin dependent type 1 diabetes (Borowiak and Melton 2009).

Finally, there are a number of neurological disorders, such as Alzheimer and Parkinson's diseases, amyotrophic lateral sclerosis and multiple sclerosis (ALS and MS respectively) as well as spinal cord injury that have been put as targets for hES cell derived therapies, and the urgency in the field of regenerative medicine is illustrated by the aforementioned clinical trials by Geron and Advanced Cell Technology as well as for neurological diseases as previously discussed by (Alper 2009; Kim and de Vellis 2009). Needless to say, the promises hES cells hold for the generation of any cell type in the human body, has spawned research efforts in many disease areas.

7.8 Conclusions and Future Development in Research

We have now had access to hES cells for more than a decade, and the progress of the scientific field has been spectacular. One could only speculate about what the next decade will generate in terms of greater understanding of human developmental biology, and all aspects of the generation of functional cells from hES cells for therapy as well as drug discovery applications. The field has started to transform from research and development phase into a state where therapeutic and industrial applications begin to be tangible. In terms of the therapeutic area, the regulatory bodies are fundamental in developing and harmonising the legal framework for the use of stem cells in the clinic, and also when it comes to drug safety assessment legislation.

The hES cell field has largely been directed by scientific drivers, however it becomes more obvious that the financial drivers become more imminent the closer to mature products we approach. Any application based on hES cells or their derivatives needs to be proven biologically effective, safe where applicable, but also economically justified, compare to the alternatives. More efficient ways of producing the appropriate qualities of cells needs to be further addressed, including defined and/or xeno-free conditions, GMP compliant,, and with the possibilities of automated production. Modified hES cells or derivatives thereof are important tools for further research and development, for example reporter gene containing hES cell lines, immortalised precursors and cells modified to overexpress genes of interest, e.g. ion channels or metabolising enzymes.

In terms of the generation of novel hES cell lines, it has been argued that there is a continued need for this. Reasons in favour for this is the technical development, that the absolute majority of the older lines are not derived under acceptable conditions for many applications, albeit the hES cell that the therapeutic cells originated from in the Geron trials were originally derived as research grade and retrospectively qualified. In order to address patient groups with various putative therapies, one can also argue that there might not be enough diversity among the existing hES cell lines to cover the needs for future cell-based therapies as well as for the development of novel drugs for treatment of disease (Civin and Rao 2006). However, studies of renal allograft donor-recipient relations give fuel for speculation that a relatively low number of hES cell lines, in the range of low hundreds or ever less, would cover the larger proportion of the population for future therapies (Nakajima et al. 2007; Taylor et al. 2005), as discussed by Daley and Scadden (2008). Some compounds are known to be metabolised differently between individuals within various ethnic backgrounds as well as between ethnic groups, one example is the well known genetic diversity of the alcohol dehydrogenase.

Arguments against further establishment of hES cell lines also include advocating alternative novel technologies such as iPS cells and genetic manipulation of major histocompatibility genes to avoid graft-host responses. Using hES cell derived cells as replacement therapy will certainly raise these issues, a potential major problem that needs to be overcome (Lui et al. 2009). From a functional perspective,

In order to develop safer and more accurate assays for drug discovery and toxicity testing there is a need of multiplicity among the hES cell lines used for the development and verification and validation of novel test systems. It has also been argued that only a few existing cell lines as well as somatic stem cells should be enough for therapeutic purposes, drug discovery research and basic science. The methods for modifying existing cell lines are developing however the concept of transplanting genetically manipulated cells spawns further regulatory questions. It is safe to say that by the date this is published, it is too early to rule out one technology on favour of another. Only time together with high quality peer reviewed research and development will contribute to the future directions of stem cells in general and hES cells in particular.

Acknowledgements The authors thank Miss Claire Balfour for providing images for the figures.

References

2001/20/EC, E.D (2001) EU Directive 2001/20/EC

2005/28/EC, E.D (2005) EU Directive 2005/28/EC

Adewumi O, Aflatoonian B, Ahrlund-Richter L, Amit M, Andrews PW, Beighton G, Bello PA, Benvenisty N, Berry LS, Bevan S et al (2007) Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol 25:803–816

Advanced Cell Technology clinical trials. http://www.advancedcell.com/patients/clinical-trial-information/

Agarwal S, Holton KL, Lanza R (2008) Efficient differentiation of functional hepatocytes from human embryonic stem cells. Stem Cells 26:1117–1127

Alper J (2009) Geron gets green light for human trial of ES cell-derived product. Nat Biotechnol 27:213–214

Andrews PD, Becroft M, Aspegren A, Gilmour J, James MJ, McRae S, Kime R, Allcock RW, Abraham A, Jiang Z, Strehl R, Mountford JC, Milligan G, Houslay MD, Adams DR, Frearson JA (2010) High-content screening of feeder-free human embryonic stem cells to identify pro-survival small molecules. Biochem J 432(1):21–33

Baharvand H, Hashemi SM, Shahsavani M (2008) Differentiation of human embryonic stem cells into functional hepatocyte-like cells in a serum-free adherent culture condition. Differentiation 76:465–477

Barde Y (2009) Caution urged in trial of stem cells to treat spinal-cord injury. Nature 458:29

Beqqali A, Kloots J, Ward-van Oostwaard D, Mummery C, Passier R (2006) Genome-wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. Stem Cells 24:1956–1967

Bongso A, Fong CY, Ng SC, Ratnam S (1994) Isolation and culture of inner cell mass cells from human blastocysts. Hum Reprod 9:2110–2117

Borowiak M, Melton DA (2009) How to make beta cells? Curr Opin Cell Biol, Dec; 21(6):727–732

Brandenberger R, Wei H, Zhang S, Lei S, Murage J, Fisk GJ, Li Y, Xu C, Fang R, Guegler K et al (2004) Transcriptome characterization elucidates signaling networks that control human ES cell growth and differentiation. Nat Biotechnol 22:707–716

Brett S, Livie M, Thomas G, McConnell A, Rajkhowa M (2009) Report on the donation of supernumerary embryos from fresh IVF and ICSI treatment cycles for human stem cell research. Hum Fertil (Camb) 12:34–39

Cai L, Johnstone BH, Cook TG, Liang Z, Traktuev D, Cornetta K, Ingram DA, Rosen ED, March KL (2007) Suppression of hepatocyte growth factor production impairs the ability of adipose-derived stem cells to promote ischemic tissue revascularization. Stem Cells 25:3234–3243

Caisander G, Park H, Frej K, Lindqvist J, Bergh C, Lundin K, Hanson C (2006) Chromosomal integrity maintained in five human embryonic stem cell lines after prolonged in vitro culture. Chromosome Res 14:131–137

Caspi O, Huber I, Kehat I, Habib M, Arbel G, Gepstein A, Yankelson L, Aronson D, Beyar R, Gepstein L (2007) Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. J Am Coll Cardiol 50:1884–1893

Catalina P, Montes R, Ligero G, Sanchez L, de la Cueva T, Bueno C, Leone PE, Menendez P (2008) Human ESCs predisposition to karyotypic instability: is a matter of culture adaptation or differential vulnerability among hESC lines due to inherent properties? Mol Cancer 7:76

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113:643–655

Chen AE, Egli D, Niakan K, Deng J, Akutsu H, Yamaki M, Cowan C, Fitz-Gerald C, Zhang K, Melton DA et al (2009) Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. Cell Stem Cell 4:103–106

Civin CI, Rao MS (2006) How many human embryonic stem cell lines are sufficient? A U.S. perspective. Stem Cells 24:800–803

Crook JM, Peura TT, Kravets L, Bosman AG, Buzzard JJ, Horne R, Hentze H, Dunn NR, Zweigerdt R, Chua F et al (2007) The generation of six clinical-grade human embryonic stem cell lines. Cell Stem Cell 1:490–494

Crook JM, Hei D, Stacey G (2010) The International Stem Cell Banking Initiative (ISCBI): raising standards to bank on. Vitro Cell Dev Biol Anim 46(3–4):169–172, Epub 3 Mar 2010. Review

D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 24:1392–1401

Daley GQ, Scadden DT (2008) Prospects for stem cell-based therapy. Cell 132:544–548

Dalgetty DM, Medine CN, Iredale JP, Hay DC (2009) Progress and future challenges in stem cell-derived liver technologies. Am J Physiol Gastrointest Liver Physiol 297:G241–G248

Desbordes SC, Placantonakis DG, Ciro A, Socci ND, Lee G, Djaballah H, Studer L (2008) High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. Cell Stem Cell 2:602–612

Dokras A, Sargent IL, Barlow DH (1993) Human blastocyst grading: an indicator of developmental potential? Hum Reprod 8:2119–2127

Draper JS, Smith K, Gokhale P, Moore HD, Maltby E, Johnson J, Meisner L, Zwaka TP, Thomson JA, Andrews PW (2004) Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol 22:53–54

Ellerstrom C, Strehl R, Moya K, Andersson K, Bergh C, Lundin K, Hyllner J, Semb H (2006) Derivation of a xeno-free human embryonic stem cell line. Stem Cells 24:2170–2176

Ellerstrom C, Strehl R, Noaksson K, Hyllner J, Semb H (2007) Facilitated expansion of human embryonic stem cells by single-cell enzymatic dissociation. Stem Cells 25:1690–1696

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156

Feki A, Bosman A, Dubuisson JB, Irion O, Dahoun S, Pelte MF, Hovatta O, Jaconi ME (2008) Derivation of the first Swiss human embryonic stem cell line from a single blastomere of an arrested four-cell stage embryo. Swiss Med Wkly 138:540–550

Gavrilov S, Prosser RW, Khalid I, MacDonald J, Sauer MV, Landry DW, Papaioannou VE (2009) Non-viable human embryos as a source of viable cells for embryonic stem cell derivation. Reprod Biomed Online 18:301–308

Geens M, Mateizel I, Sermon K, De Rycke M, Spits C, Cauffman G, Devroey P, Tournaye H, Liebaers I, Van de Velde H (2009) Human embryonic stem cell lines derived from single blastomeres of two 4-cell stage embryos. Hum Reprod 24:2709–2717

Geron (2009) Geron receives FDA clearance to begin world's first human clinical trial of embryonic stem cell-based therapy. Press release

Guguen-Guillouzo C, Corlu A, Guillouzo A (2010) Stem cell-derived hepatocytes and their use in toxicology. Toxicology 270(1):3–9, Epub 6 Oct 2009

Heins N, Englund MC, Sjoblom C, Dahl U, Tonning A, Bergh C, Lindahl A, Hanson C, Semb H (2004) Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells 22:367–376

Heiskanen A, Satomaa T, Tiitinen S, Laitinen A, Mannelin S, Impola U, Mikkola M, Olsson C, Miller-Podraza H, Blomqvist M et al (2007) N-glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. Stem Cells 25:197–202

Ilic D, Stephenson E, Wood V, Jacquet L, Stevenson D, Petrova A, Kadeva N, Codognotto S, Patel H, Semple M, Cornwell G, Ogilvie C, Braude P (2012) Derivation and feeder-free propagation of human embryonic stem cells under xeno-free conditions. Cytotherapy 14(1):122–128, Epub 27 Oct 2011

Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N (2000) Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 6:88–95

Jensen J, Hyllner J, Bjorquist P (2009) Human embryonic stem cell technologies and drug discovery. J Cell Physiol 219:513–519

Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L (2001) Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 108:407–414

Kim SU, de Vellis J (2009) Stem cell-based cell therapy in neurological diseases: a review. J Neurosci Res 87:2183–2200

Klimanskaya I, Chung Y, Becker S, Lu SJ, Lanza R (2006) Human embryonic stem cell lines derived from single blastomeres. Nature 444:481–485

Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S et al (2007) Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25:1015–1024

Leor J, Gerecht S, Cohen S, Miller L, Holbova R, Ziskind A, Shachar M, Feinberg MS, Guetta E, Itskovitz-Eldor J (2007) Human embryonic stem cell transplantation to repair the infarcted myocardium. Heart 93:1278–1284

Lerou PH, Yabuuchi A, Huo H, Miller JD, Boyer LF, Schlaeger TM, Daley GQ (2008a) Derivation and maintenance of human embryonic stem cells from poor-quality in vitro fertilization embryos. Nat Protoc 3:923–933

Lerou PH, Yabuuchi A, Huo H, Takeuchi A, Shea J, Cimini T, Ince TA, Ginsburg E, Racowsky C, Daley GQ (2008b) Human embryonic stem cell derivation from poor-quality embryos. Nat Biotechnol 26:212–214

Li X, Krawetz R, Liu S, Meng G, Rancourt DE (2009) ROCK inhibitor improves survival of cryopreserved serum/feeder-free single human embryonic stem cells. Hum Reprod 24:580–589

Lim JW, Bodnar A (2002) Proteome analysis of conditioned medium from mouse embryonic fibroblast feeder layers which support the growth of human embryonic stem cells. Proteomics 2(9):1187–1203

Loser P, Schirm J, Guhr A, Wobus AM, Kurtz A (2010) Human embryonic stem cell lines and their use in international research. Stem Cells 28:240–246

Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, Crandall LJ, Daigh CA, Conard KR, Piekarczyk MS et al (2006) Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 24:185–187

Lui KO, Waldmann H, Fairchild PJ (2009) Embryonic stem cells: overcoming the immunological barriers to cell replacement therapy. Curr Stem Cell Res Ther 4(1):70–80

Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 78:7634–7638

Martin MJ, Muotri A, Gage F, Varki A (2005) Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med 11:228–232

Medine CN, Lucendo-Villarin B, Zhou W, West CC, Hay DC (2011) Robust generation of hepatocyte-like cells from human embryonic stem cell populations. J Vis Exp 56:e2969. doi:10.3791/2969

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113:631–642

Mummery C, Ward-van Oostwaard D, Doevendans P, Spijker R, van den Brink S, Hassink R, van der Heyden M, Opthof T, Pera M, de la Riviere AB et al (2003) Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation 107:2733–2740

Murdoch A, Braude P, Courtney A, Brison D, Hunt C, Lawford-Davies J, Moore H, Stacey G, Sethe S, for the Procurement Working Group of the National Clinical hESC Forum (2012) The procurement of cells for the derivation of human embryonic stem cell lines for therapeutic use: recommendations for good practice. Stem Cell Rev 8(1):91–99

Nakajima F, Tokunaga K, Nakatsuji N (2007) Human leukocyte antigen matching estimations in a hypothetical bank of human embryonic stem cell lines in the Japanese population for use in cell transplantation therapy. Stem Cells 25:983–985

Nasonkin IO, Koliatsos VE (2006) Nonhuman sialic acid Neu5Gc is very low in human embryonic stem cell-derived neural precursors differentiated with B27/N2 and noggin: implications for transplantation. Exp Neurol 201:525–529

Parsons XH, Teng YD, Parsons JF, Snyder EY, Smotrich DB, Moore DA (2011) Efficient derivation of human cardiac precursors and cardiomyocytes from pluripotent human embryonic stem cells with small molecule induction. J Vis Exp 57:e3274. doi:10.3791/3274

Passier R, Mummery C (2005) Cardiomyocyte differentiation from embryonic and adult stem cells. Curr Opin Biotechnol 16:498–502

Prowse AB, McQuade LR, Bryant KJ, Van Dyk DD, Tuch BE, Gray PP (2005) A proteome analysis of conditioned media from human neonatal fibroblasts used in the maintenance of human embryonic stem cells. Proteomics 5(4):978–989

Rajala K, Hakala H, Panula S, Aivio S, Pihlajamaki H, Suuronen R, Hovatta O, Skottman H (2007) Testing of nine different xeno-free culture media for human embryonic stem cell cultures. Hum Reprod 22:1231–1238

Rambhatla L, Chiu CP, Kundu P, Peng Y, Carpenter MK (2003) Generation of hepatocyte-like cells from human embryonic stem cells. Cell Transplant 12:1–11

Rho JY, Yu K, Han JS, Chae JI, Koo DB, Yoon HS, Moon SY, Lee KK, Han YM (2006) Transcriptional profiling of the developmentally important signalling pathways in human embryonic stem cells. Hum Reprod 21:405–412

Richards M, Fong CY, Tan S, Chan WK, Bongso A (2004) An efficient and safe xeno-free cryopreservation method for the storage of human embryonic stem cells. Stem Cells 22:779–789

Sjogren A, Hardarson T, Andersson K, Caisander G, Lundquist M, Wikland M, Semb H, Hamberger L (2004) Human blastocysts for the development of embryonic stem cells. Reprod Biomed Online 9:326–329

Sjogren-Jansson E, Zetterstrom M, Moya K, Lindqvist J, Strehl R, Eriksson PS (2005) Large-scale propagation of four undifferentiated human embryonic stem cell lines in a feeder-free culture system. Dev Dyn 233:1304–1314

Snykers S, De Kock J, Rogiers V, Vanhaecke T (2009) In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. Stem Cells 27:577–605

Stephenson EL, Braude PR, Mason C (2007) International community consensus standard for reporting derivation of human embryonic stem cell lines. Regen Med 2:349–362

Strelchenko N, Verlinsky O, Kukharenko V, Verlinsky Y (2004) Morula-derived human embryonic stem cells. Reprod Biomed Online 9:623–629

Suemori H, Yasuchika K, Hasegawa K, Fujioka T, Tsuneyoshi N, Nakatsuji N (2006) Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. Biochem Biophys Res Commun 345:926–932

Synnergren J, Akesson K, Dahlenborg K, Vidarsson H, Ameen C, Steel D, Lindahl A, Olsson B, Sartipy P (2008) Molecular signature of cardiomyocyte clusters derived from human embryonic stem cells. Stem Cells 26:1831–1840

Taylor CJ, Bolton EM, Pocock S, Sharples LD, Pedersen RA, Bradley JA (2005) Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. Lancet 366:2019–2025

Thomas RJ, Anderson D, Chandra A, Smith NM, Young LE, Williams D, Denning C (2009) Automated, scalable culture of human embryonic stem cells in feeder-free conditions. Biotechnol Bioeng 102:1636–1644

Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, Hearn JP (1995) Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci USA 92:7844–7848

Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Hearn JP (1996) Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. Biol Reprod 55:254–259

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147

Unger C, Skottman H, Blomberg P, Dilber MS, Hovatta O (2008) Good manufacturing practice and clinical-grade human embryonic stem cell lines. Hum Mol Genet 17:R48–R53

Uosaki H, Fukushima H, Takeuchi A, Matsuoka S, Nakatsuji N, Yamanaka S, Yamashita JK (2011) Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by VCAM1 surface expression. PLoS One 6(8):e23657, Epub 18 Aug 2011

van Laake LW, Passier R, Monshouwer-Kloots J, Verkleij AJ, Lips DJ, Freund C, den Ouden K, Ward-van Oostwaard D, Korving J, Tertoolen LG et al (2007) Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. Stem Cell Res 1:9–24

van Laake LW, Passier R, den Ouden K, Schreurs C, Monshouwer-Kloots J, Ward-van Oostwaard D, van Echteld CJ, Doevendans PA, Mummery CL (2009) Improvement of mouse cardiac function by hESC-derived cardiomyocytes correlates with vascularity but not graft size. Stem Cell Res 3:106–112

van Laake LW, van Donselaar EG, Monshouwer-Kloots J, Schreurs C, Passier R, Humbel BM, Doevendans PA, Sonnenberg A, Verkleij AJ, Mummery CL (2010) Extracellular matrix formation after transplantation of human embryonic stem cell-derived cardiomyocytes. Cell Mol Life Sci 67(2):277–290, Epub 22 Oct 2009

Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Muguruma K, Sasai Y (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 25:681–686

Xu RH, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP, Thomson JA (2002) BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat Biotechnol 20:1261–1264

Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM et al (2008) Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature 453:524–528

Zhang X, Stojkovic P, Przyborski S, Cooke M, Armstrong L, Lako M, Stojkovic M (2006) Derivation of human embryonic stem cells from developing and arrested embryos. Stem Cells 24:2669–2676

Chapter 8
Induced Pluripotent Stem Cells

Keisuke Okita, Kazutoshi Takahashi, and Shinya Yamanaka

Abstract Nearly 50 years have passed since the concept of nuclear reprogramming proposed for the first time. Since then, several approaches have been developed to convert somatic cells to a pluripotent state. Direct reprogramming with defined factors, is the newest of these approaches. This method requires just a few genes, and it also has a great reproducibility. Applying this method to humans seems to open the door to cell transplantation therapy without immune rejection, drug discovery, and elucidation of the pathogenesis of intractable diseases.

However, this concept still faces some issues which must be overcome before application due to a shortage of experience. This chapter introduces an overview of direct reprogramming as well as a special focus on its potentials and challenges.

8.1 Introduction

Pluripotent stem cells such as embryonic stem (ES) cells may be a hopeful resource for regenerative medicine to repair degenerative or damaged tissues. ES cells have the potential to differentiate into all cell types in the body including germ cells. Not only pluripotency but also their growth properties are substantially superior. In conventional

K. Okita, Ph.D. • K. Takahashi, Ph.D.
Center for iPS Cell Research and Application(CiRA),
Institute for Integrated Cell-Material Sciences, Kyoto University,
53 Kawahara-cho Shogoin Sakyo-ku, Kyoto 606-8507, Japan

S. Yamanaka, M.D., Ph.D. (✉)
Center for iPS Cell Research and Application(CiRA),
Institute for Integrated Cell-Material Sciences, Kyoto University,
53 Kawahara-cho Shogoin Sakyo-ku, Kyoto 606-8507, Japan

S.Yamanaka is a member without salary of the scientific advisory boards of iPierian, iPS Academia Japan, Megakaryon Corporation, and Retina Institute Japan
e-mail: yamanaka@cira.kyoto-u.ac.jp

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_8,

conditions, ES cells can grow infinitely while maintaining an undifferentiated state. Combined with the techniques of gene targeting and transgenes, these characters have allowed the generation of genetically modified animals (Robertson et al. 1986; Thomas and Capecchi 1987; Doetschman et al. 1987). To this end, the mechanisms or causes of many diseases have been elucidated. Reports on the establishment of human ES cells brought great expectations to regenerative medicine of various diseases (Thomson et al. 1998). In the last decade, scientists have established appropriate culture conditions, differentiation protocols and guidelines for users.

On the other hand, human ES cells face two big issues that need to be overcome before successful medical application can be achieved. One is immune rejection caused by the mismatch in human leukocyte antigen (HLA) haplotypes between ES cells and patient. Another is the usage of human embryos. Although the response to ethical issues may vary depending on regions and civilizations, it should be earnestly addressed in any case.

One of the solutions to overcome these issues is to reprogram patient's own somatic cells directly to pluripotent stem cells. The reprogramming of the somatic nucleus was firstly demonstrated by Sir John Gurdon in 1958 (Gurdon et al. 1958). He developed cloned frogs by injecting the nucleus of a tadpole somatic cell into an egg. This report suggested that frog eggs have some reprogramming factor(s) that could initialize somatic state back to totipotency. In 1997, the group of Sir Ian Wilmut and Dr. Keith Campbell with their famous sheep, Dolly, showed that not only flexible species such as amphibians but also mammals have the reprogramming factor(s) in their eggs by using nuclear transfer technology (Wilmut et al. 1997). In the last year of the 20th century, Dr. Takashi Tada and his colleagues showed that the nucleus of somatic cell also could be reset to the pluripotent state by electrical-fusion with mouse ES cells (Tada et al. 2001). All these findings and following associated reports suggested that ES cells as well as eggs have such reprogramming factor(s). At a later date, the same phenomenon was confirmed in human cells (Cowan et al. 2005).

What is the reprogramming factor? People have hypothesized that factors playing important roles in ES cell identities, such as differentiation potentials and tumor-like growth properties, play a crucial role in the induction of pluripotency in somatic cells. The concept that the genes expressed specifically in stem cells provided multipotency to the cells has been established and called stemness (Ramalho-Santos et al. 2002). Therefore, the genes expressed predominantly in eggs and/or ES cells, and some non-ES cell specific genes which are important for the character of pluripotent cells can be candidates of reprogramming factor(s).

The importance of pluripotent cell-associated genes has been proposed in several studies (Boiani and Schöler 2005; Niwa 2007).

Oct3/4 (also known as Pou5f1) one of the most famous ES cell-specific genes, is an octamer sequence binding transcription factor (Okamoto et al. 1990; Schöler et al. 1990). Deletion of *Oct3/4* gene caused the loss of pluripotency in both ES cells and early embryos (Nichols et al. 1998). *Oct3/4*-null embryos die at around the implantation stage. The inner cell mass (ICM) of *Oct3/4*-deficient embryo can no longer outgrow. Detailed analyses have shown that Oct3/4 prevents the differentiation into trophectoderm by suppressing the function of Cdx2 which plays essential role in trophectoderm development (Niwa et al. 2005). On the other hand, only a

1.5-fold increase of Oct3/4 expression in ES cells is sufficient to trigger differentiation into either mesoderm or primitive endoderm (Niwa et al. 2000). These data indicated that Oct3/4 is one of the most important regulators to prevent differentiation into specific lineages and maintain ES cells in a pluripotent state.

The other important player is Sox2. The expression of Sox2 is restricted in nerve tissue and pluripotent cells including germ cells (Kamachi et al. 2000). Structural and biochemical analyses demonstrated that Sox2 binds directly to Oct3/4 and acts as a regulator of ES cell-specific gene expression such as *Utf1*, *Fgf4* and *Lefty1* (Ambrosetti et al. 1997; Reményi et al. 2003; Nishimoto et al. 1999). Homozygous deletion of *Sox2* gene leads to embryo death immediate after implantation with the lack of epiblast formation (Avilion et al. 2003). Blastocysts carrying a null mutation of *Sox2* gene looked normal, but the ICM could not expand *in vitro* whereas trophoblasts and primitive endoderm cells can continue to proliferate. In addition, Sox2-deficient ES cells cannot maintain an undifferentiated state in conventional conditions (Masui et al. 2007). Taken together, Sox2 is crucial for the maintenance of pluripotent cells in both ES cells and early embryos.

However, the functions of Oct3/4 and Sox2 are not sufficient to explain the molecular mechanisms underlying pluripotent stem cell identity. The presence of other co-factors has been speculated. Many exhaustive analyses were performed in the early 2000s, and databases of gene expression profiles were advanced rapidly such as expressed sequence tags (ESTs) and microarray technology (Kawai et al. 2001). To this end, hundreds of stemness-relating genes were found as candidates to define the stem cell phenotype (Tokuzawa et al. 2003; Takahashi et al. 2003). An *in silico* analysis and functional screening identified a novel stemness gene, designated Nanog at the same time (Mitsui et al. 2003). Nanog is a transcription factor which contains a paired-like homeobox. The expression pattern of Nanog in early development is more restricted than that of Oct3/4 or Sox2. Embryos carrying a null-mutation of Nanog gene die during the post-implantation period lacking epiblasts (Mitsui et al. 2003). Outgrowth of Nanog-deficient ICM is also defective, although primitive endoderm-like cells can proliferate. On the other hand, ES cells carrying homozygous deletion of the Nanog gene can proliferate in vitro although morphologies and gene expression patterns were changed to primitive endoderm-like state (Mitsui et al. 2003). In addition, forced expression of the Nanog gene allows cells to maintain pluripotency even without leukemia inhibitory factor (LIF) which is an essential component for self-renewal of mouse ES cells in serum-containing medium (Chambers et al. 2003; Mitsui et al. 2003). Therefore, these data suggested that Nanog acts as a switch between the pluripotent state and primitive endoderm.

ChIP on chip analyses, which combine chromatin immunoprecipitation, microarray, and a transcriptome, revealed global target genes of Oct3/4, Sox2 and Nanog in ES cells (Chew et al. 2005; Loh et al. 2006; Boyer et al. 2005). More than 300 genes were categorized as common targets of these three transcription factors, including both expressed (Oct3/4, Sox2, Nanog, Stat3, Zic3) and not expressed genes (Hoxb1, Pax6, Lhx5, Myf5) in ES cells. These data suggested that a circuit consisting of Oct3/4, Sox2 and Nanog promotes the expression of genes supporting the self-renewal of ES cells, suppresses those genes required for differentiation into three germ layers such as homeobox genes, and thus maintains pluripotency.

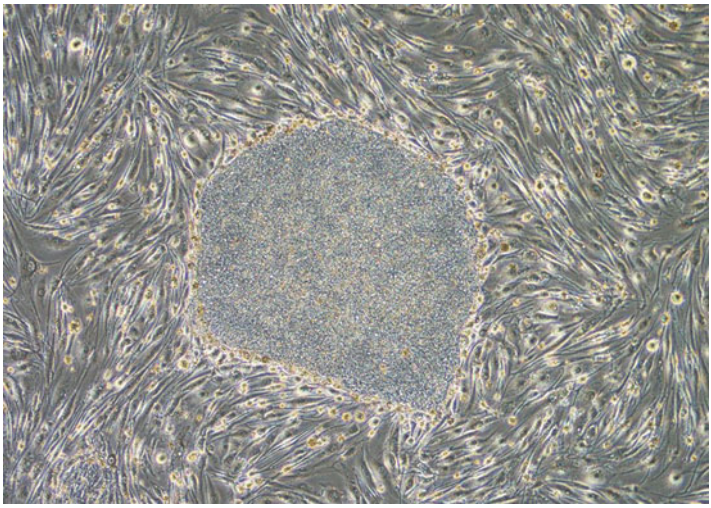


Fig. 8.1 iPS cells derived from adult human dermal fibroblasts

On the other hand, several studies reported that some oncogenes also played important roles in ES cell identity (Chambers and Smith 2004; Cheng et al. 1998). The most famous oncogene related to self-renewal of mouse ES cells is Stat3. LIF-mediated Stat3 activation is essential and sufficient for the maintenance of pluripotency (Niwa et al. 1998). One of its downstream targets in LIF/Stat3 signaling pathway is c-Myc (Cartwright et al. 2005). An overexpression of c-Myc in mouse ES cells allows LIF-independent self-renewal. On the other hand, another well known oncogenic pathway, Ras/MAPK, negatively regulates the maintenance of pluripotency. Inhibition of the Ras/MAPK pathway by a small molecule or gene targeting blocks differentiation (Cheng et al. 1998; Burdon et al. 1999). It is not always true that these factors are expressed specifically in pluripotent cells. ES cells display tumor-like properties with regard to their growth including anchorage independence, infinite expansion and tumorigenicity. As a result, it is no wonder that tumor-related genes join the network of pluripotency.

In 2006, direct reprogramming of mouse somatic cells was accomplished by introducing the combination of just four transcription factors; Oct3/4, Sox2, Klf4 and c-Myc (Takahashi and Yamanaka 2006). These reprogrammed cells artificially induced by the defined factors were named induced Pluripotent Stem (iPS) cells. In 1 year, human iPS cells were also generated from adult dermal fibroblasts (Fig. 8.1) (Takahashi et al. 2007; Yu et al. 2007). Since the first announcement of these findings, this field has rapidly expanded and developed in various directions.

In this chapter, I will introduce the expected potential and possible problems of iPS cells based on the latest findings.

8.2 Derivation

8.2.1 Cell Sources

In mouse, MEFs and TTFs are commonly used as sources of iPS cells in many reports. Various other cell types were demonstrated to be sources of iPS cells, such as neural progenitors, adrenal glands, keratinocytes, muscular cells, intestinal epithelium cells, mesenchymal stem cells and hematopoietic cells (Aoi et al. 2008; Wernig et al. 2008a; Silva et al. 2008; Kim et al. 2008; Eminli et al. 2008, 2009). In addition, terminally differentiated cells such as mature B cells, T cells and pancreatic β-cells can also be reprogrammed into iPS cells (Hong et al. 2009; Hanna et al. 2008; Stadtfeld et al. 2008a).

In human, fibroblasts derived from a fetus, neonatal foreskin, oral mucosa, and adult dermis are widely used (Takahashi et al. 2007; Yu et al. 2007). Human iPS cells were also generated from keratinocytes, mesenchymal stroma cells and less invasive cells, such as amnion cells, umbilical cord blood cells, dental pulp stem cells, and peripheral blood mononuclear cells (Aasen et al. 2008; Loh et al. 2009; Ye et al. 2009; Giorgetti et al. 2009; Haase et al. 2009; Yan et al. 2010). The efficiency of iPS cell generation and methods to deliver reprogramming factors are largely dependent on the cell types.

iPS cell inductions were also reported from rat, dog, rabbit, pig, horse, sheep, cattle and monkey (Liao et al. 2009; Shimada et al. 2010; Esteban et al. 2009; Honda et al. 2010; Liu et al. 2008; Nagy et al. 2011; Sumer et al. 2011; Li et al. 2011). The technique also applied for the generation of iPS cells from endangered species: the drill, *Mandrillus leucophaeus* and the northern white rhinoceros, *Ceratotherium simum cottoni*. These iPS cells might be helpful to save species (Ben-Nun et al. 2011).

8.2.2 Reprogramming Factors and Substitutes

Mouse iPS cells were initially established by forced-expression of Oct3/4, Sox2, Klf4 and c-Myc in mouse embryonic fibroblasts (MEFs) or tail-tip fibroblasts (TTFs) from adult mice (Takahashi and Yamanaka 2006). Some of these factors can be replaced with related genes. For example, Klf2 or Klf5 can mimic Klf4 functions, and Sox1, Sox3, Sox15, Sox17 and Sox18 are also able to substitute for Sox2 (Nakagawa et al. 2008). Estrogen-related receptor, beta (Esrrb), can contribute to direct reprogramming with Oct3/4 and Sox2 (Feng et al. 2009). In addition, treatment with a chemical drug, kenpaullone, along with transduction of Oct3/4 and Sox2 also can produce ES-like colonies from MEFs (Lyssiotis et al. 2009).

All of the Myc family genes in mammals, c-Myc, N-Myc and L-Myc, dramatically enhance the efficiency of iPS cell generation (Nakagawa et al. 2008). Although Myc is dispensable for direct reprogramming, the number of iPS cell colonies generally

diminishes to approximately 150 without Myc (Nakagawa et al. 2008; Wernig et al. 2008b). These data suggest that Myc acts as a booster of direct reprogramming. The effects of Myc on iPS cell generation can be replaced by the activation of the canonical Wnt pathway (Marson et al. 2008).

In addition to the above, some other factors which can improve the reprogramming efficiency were identified. The transduction of microRNAs such as miR-291-3p, miR-294, miR-295 elevates the number of iPS cell colonies by about tenfold (Judson et al. 2009). In addition, RNA-related protein LIN28 enhances the efficiency of human iPS cells generation (Yu et al. 2007; Liao et al. 2008). The inhibition of both Mitogen-Activated Protein Kinase Kinase (MEK) and Glycogen synthase kinase (GSK) 3, which is generally called 2i, increases the reprogramming efficiency (Silva et al. 2008). The overexpression of Spalt-like 4 (Sall4) or the suppression of transformation related protein 53 (Trp53) increase the reprogramming efficiency in both mouse and human (Tsubooka et al. 2009; Hong et al. 2009; Kawamura et al. 2009; Li et al. 2009; Marión et al. 2009; Utikal et al. 2009; Banito et al. 2009). In the case of human cells, telomerase reverse transcriptase (TERT) and Simian virus 40 large T (SV40LT) antigen that promote the immortalization of human primary fibroblasts, can enhance the reprogramming efficiency (Park et al. 2008b; Mali et al. 2008). Accumulating evidences have revealed many additional reprogramming factors, such as GLIS1, UTF1, NR5A2, and RARG (Maekawa et al. 2011; Wang et al. 2011; Zhao et al. 2008).

The expression balance and timing of the reprogramming factors is also important for iPS cell generation. Increment of exogenous OCT3/4 enhanced reprogramming frequency whereas relatively higher expression of SOX2, KLF4 or C-MYC inhibited the efficiency (Papapetrou et al. 2009). The balance of transgene expression would also affect the quality of iPS cells (Carey et al. 2011).

8.2.3 Reprogramming Methods

First iPS cells were generated by transduction with four reprogramming factors with retroviral vectors (Takahashi and Yamanaka 2006). This method has proven to be an effective one. However, viral integration into the genome should be avoided for clinical applications, because of unexpected activation of oncogenic adjacent genes and/or transgenes or destruction of genome, which may result in tumor formation (Hacein-Bey-Abina et al. 2003; Okita et al. 2007). Many attempts have been reported to overcome the issue. Soldner and colleagues used lentiviral vectors including the loxP sequence in their 3′ long terminal repeat (LTR) (Soldner et al. 2009). When lentiviruses integrated into the genome, most of the expression units are flanked by two loxP. After isolation of iPS cell clones, the sequence flanked by loxP can be removed by Cre recombinase. Only a ~100 bp sequence, which is a part of the LTR, should be left due to the limitations of this concept.

The reported approach to make integration-free iPS cells can be divided into four categories based on their vector types; virus, DNA, RNA, and protein. The

generation of iPS cells using an integration-free method was initially reported in mice. Stadtfeld et al. and Okita et al. used adenoviruses and plasmids to deliver the reprogramming factors, respectively (Stadtfeld et al. 2008b; Okita et al. 2008). Both adenoviruses and plasmids are diluted by cell division and finally disappear. Yu et al. showed that integration-free human iPS cells could be established by plasmid vectors containing Epstein-Barr virus (EBV) sequences (Yu et al. 2009). The EBV vectors encodes EBV nuclear antigen 1 (EBNA1) which enable to replicate the vector in human cells and to maintain them episomally. Therefore, the long-term expression of transgenes is guaranteed. On the other hand, the replication efficiency is not 100%. Therefore the vectors are gradually lost after iPS cell generation. They confirmed that no transgenes were inserted in the genome of iPS clones by Southern blotting and PCR. However, small pieces of exogenous DNA may slip away in the genome. This must be further analyzed in detail by next-generation sequencing. In addition, the low efficiency of reprogramming with transient expression is another serious problem. This could be overcome by improvement of the vector and optimization of reprogramming factors (Okita et al. 2011). The efficiencies are around 0.1%, which is less than those of virus-mediated methods (1%), but would be enough for many purposes.

Kaji et al., Woltjen et al. and Yusa et al. pursued another path to generate integration-free iPS cells. They chose the transposon system, *piggybac*, to introduce a set of reprogramming factors into the genome (Kaji et al. 2009; Woltjen et al. 2009; Yusa et al. 2009). It is particularly noteworthy that *piggybac* can be removed from the genome in a seamless manner by transposase when the reprogramming events are over. This system includes the forthcoming ablation of transgenes unlike other methods with transient expression of transposase. On the other hand, because *piggybac* has to insert once into the genome of somatic cells, it is necessary to confirm that there is no footprint in the integration sites after excision. Moreover, no clinical trials using transposon have so far been conducted.

All of these methods are based on the expression units of reprogramming factors. In contrast, recombinant protein fused with poly-arginine, which permeates into the target cells could achieve generation of mouse iPS cells (Zhou et al. 2009). They performed the forced expression of poly-arginine tagged Oct3/4, Sox2, Klf4 and c-Myc in *E. coli*, and then purified them. They transduced these proteins to MEF for four times every other day. ES-like colonies appeared after 30 days incubation, and grew into transgene-free iPS cells. They demonstrated these protein-induced pluripotent cell lines could not only differentiate into three germ layers *in vitro* but also contribute to germ cells of chimeric mice. Human fibroblasts were also reprogrammed by protein transduction (Kim et al. 2009). They used crude extracts of the cells expressing poly-arginine tagged reprogramming factors. Its efficiency, however, seemed to be very low.

Generation of iPS cells with RNA virus vectors is also reported. Fusaki et al. and Nishimura et al. constructed Sendai virus vector which encode reprogramming factors, and generated iPS cells from human fibroblasts and peripheral T cells (Fusaki, et al. 2009; Nishimura et al. 2011; Seki et al. 2010). As the RNA genome of the Sendai virus

is maintained only in cytoplasm, they do not have chance to integrate into host genome. Sendai virus is not pathogenic to human and has relatively broad host range.

Direct induction of synthesized RNA is also available to make iPS cells. Warren et al. prepared synthesized mRNAs encoding five reprogramming factors (Oct3/4, Sox2, Klf4, c-Myc, and LIN28) with modified residues of 5-methylcytidine triphosphate and pseudouridine triphosphate to suppress the activation of the cellular antiviral response (Warren et al. 2010). In addition, a 5′ guanine cap was incorporated by inclusion of a synthetic cap analog to increase the RNA half-life in the cytoplasm. After purification, the RNAs were daily transfected into human fibroblasts and induced iPS cells. On the other hand, Miyoshi et al. established iPS cells from mouse and human somatic cells with the combination of mature microRNAs, mir-200c and families of mir-302s and mir-369s (Miyoshi et al. 2011). These RNA-based methods would provide non-integrated iPS cells.

8.2.4 Selection of Reprogrammed Cells

Generally, mice carrying a reporter system of fluorescent proteins and/or drug resistance genes driven by the promoters of pluripotent cell-associated genes are used for the establishment of mouse iPS cells. First, iPS cells are generated from MEFs or TTFs carrying *βgeo*, which is a beta-galactosidase and neomycin resistance fusion gene knocked into the *Fbx15* locus (Takahashi and Yamanaka 2006). Thereafter, other reporter systems have been designed for this purpose. Nanog or Oct3/4 can also work as selection markers of reprogramming (Okita et al. 2007; Wernig et al. 2007; Maherali et al. 2007). The other indicator of reprogramming is the silencing of the retrovirus promoter (Nakagawa et al. 2008). The long terminal repeat (LTR) of mouse molony-leukemia virus (MMLV) can act as a strong promoter in mouse fibroblasts. In contrast, the activity of MMLV LTR is effectively silenced in pluripotent cells such as ES cells and iPS cells. Although this phenomenon would involved in epigenetic modification of histone and DNA, the precise underlying mechanisms still remain unclear (Wolf and Goff 2007, 2009; Matsui et al. 2010). Nevertheless, when a retrovirus encoding fluorescent protein is transduced along with the reprogramming factors into somatic cells, the disappearance of the fluorescence suggests that reprogramming has been completed.

These reporters are employed because it is quite hard to distinguish reprogrammed cells from the non-reprogrammed cells just by morphology in mice. In contrast, there is little unrest for human cells. Human iPS cells form distinctive flat, tightly packed and clear edged colonies like human ES cells, whereas non-reprogrammed cells show granular morphologies and tend to form rough colonies (Takahashi et al. 2007; Lowry et al. 2008). Most human iPS cells have been established based on only their morphology. However, there are several attempts to use reporter system to select better iPS cells. For example, transduction of EGFP reporter with tandem repeat sequence responsible for OCT3/4 or NANOG was accomplished by lentivirus vector (Hotta et al. 2009).

8.3 Epigenetics

Some reports have shown that treatment with histone deacetylase (HDAC) inhibitors, such as Trichostatin A (TSA) and valproic acid (VPA), and DNA methyltransferase (DNMT) inhibitor, 5-Aza-2′-deoxycytidine, can improve the frequency of iPS cell establishment (Huangfu et al. 2008a, b; Mikkelsen et al. 2008). Inhibition of histone methyltransferase G9a by treatment with a small molecule, BIX-01294, is also effective. These data suggest that epigenetic modifications are closely linked to nuclear reprogramming (Shi et al. 2008).

Several studies have suggested that the process of iPS cell generation contains stochastic events, and it results in the variation of epigenetic modification, especially when they are not directly correlated with the maintenance of iPS cell state. In mouse studies, Kim et al. reported that iPS cells in early passage remains inherited epigenetic status of their cell source, and have high differentiation potential into their original cell linage (Kim et al. 2010). Some of the persisted status, however, seemed to be gradually erased during cultivation. Epigenetic inheritance is also observed in human cells (Bar-Nur et al. 2011). Global epigenetic survey found genomic regions which showed high variation among iPS clones (Lister et al. 2011). These differences would influence the character of iPS cells, in terms of differentiation potential and safety aspect.

8.4 Properties

The characteristics of mouse iPS cells are almost completely equivalent to those of mouse ES cells. The morphology of both mouse ES cells and iPS cells is identical. Those two cell types can grow in serum-containing medium in the presence of leukemia inhibitory factor (LIF) and/or feeder cells (Okita et al. 2007). They can also be maintained in serum-free medium supplemented 2i (Silva et al. 2008). In addition, iPS cells also have the potential to undergo homologous recombination that is a useful property of ES cells in biology (Hanna et al. 2007).

The expression levels of pluripotent cell marker genes such as *ERas*, *Rex1* and *Esg1* are indistinguishable between ES cells and iPS cells. Microarray analyses have also shown the global gene expression patterns of mouse iPS cells to be very similar to those of mouse ES cells (Okita et al. 2007; Wernig et al. 2007; Maherali et al. 2007).

Not only the transcripts but the epigenetic status of iPS cells and ES cells, including DNA methylation and histone modification, are quite similar (Meissner et al. 2008). CpG methylation in the promoter regions of pluripotent cell marker genes such as *Oct3/4*, *Rex1* and *Nanog* are highly unmethylated in iPS cells, whereas those of MEFs or TTFs were steadily methylated. Previous reports demonstrated that both the 4th (K4) and 27th (K27) lysine residues of histone H3 are methylated around the locus of differentiation-associated genes such as *Gata4*, *Pax6* and *Msx2* in mouse ES cells (Bernstein et al. 2006). Generally, K4 is methylated in the regions

transcriptionally activated. In contrast, methylation of K27 reflects silencing in the vicinity. These bivalent patterns of histone methylation may imply that ES cells are always ready and waiting to initiate differentiation. In addition, mouse iPS cells also show bivalent patterns of histone methylation in differentiation marker genes as similar to mouse ES cells (Maherali et al. 2007). Therefore, in the broad view of both external and inner aspects, it seems that iPS and ES cells have a striking resemblance. However, detailed analyses suggest that the epigenetic patterns are not completely identical between ES cells and iPS cells (Mikkelsen et al. 2008).

These similarities and differences are also found in human cells. In particular, the patterns of global DNA methylation differ between human iPS cells and human ES cells (Chin et al. 2009). On the other hand, various differences such as gene expression patterns, statuses of X-chromosome inactivation and differentiation capacities have been reported even among human ES clones (International Stem Cell Initiative 2007). One of the conceivable reasons for this is the variety of genetic backgrounds among humans, unlike the situation regarding experimental animals. In fact, ES clones derived from different blastocysts provided by the same couple have been shown to have a strong resemblance to each other (Chen et al. 2009).

8.5 Differentiation Capacity and Their Precursors

Many protocols for *in vitro* differentiation of mouse ES cells have been established. Most of them can be applied to mouse iPS cells. The traditional method with embryoid body formation allows iPS cells to differentiate into all three germ layers of endoderm, mesoderm and ectoderm (Takahashi and Yamanaka 2006; Wernig et al. 2007; Maherali et al. 2007). *In vitro* differentiations of iPS cells into specific cell types such as cardiac cells, bloods, adipocytes and retinal epitheliums has also been achieved (Schenke-Layland et al. 2008; Narazaki et al. 2008; Tashiro et al. 2009; Senju et al. 2009). The gold standard to test the pluripotency is the generation of chimeric mice and the subsequent germ-line transmission. Similar to ES cells, mouse iPS cells are able to contribute to the development of chimeric mice including germ cells (Okita et al. 2007; Wernig et al. 2007; Maherali et al. 2007). The successful rate of germ-line transmission for iPS cells is no less than that for ES cells. In addition, mouse iPS cells have hurdled the stricter challenge of tetraploid complementation (Kang et al. 2009; Zhao et al. 2009; Boland et al. 2009). The tetraploid embryos generated by the fusion of two blastomeres can contribute only to extra embryonic tissues instead of pup body. When iPS cells are injected into a tetraploid blastocyst, the entire body of the pup should be derived from the injected iPS cells. The pups only from iPS cells are then successfully born. These results suggest that mouse iPS cells have already reached the pluripotency of ES cells.

Most of the protocols for human ES cell differentiation are also applicable to human iPS cells. Random differentiation of human iPS cells can be achieved by embryoid body formation *in vitro* or teratoma experiments *in vivo* (Takahashi et al. 2007; Yu et al. 2007). Directed differentiation into specific cell lineages has also

succeeded, such as differentiation into retinal cells, vascular cells, pancreatic insulin-producing cells, hepatocyte-like cells, functional cardiomyocytes, platelets and neuronal cells (Osakada et al. 2009; Viczian et al. 2009; Taura et al. 2009; Zhang et al. 2009a, b; Song et al. 2009; Gai et al. 2009; Karumbayaram et al. 2009; Chambers et al. 2009; Takayama et al. 2010).

8.6 Potential Applications for Therapies

One of the potentially useful applications of iPS cell technology is that the source of regenerative medicine using a patient's own pluripotent stem cells (Yamanaka 2009). These taylor-made iPS cells would thus make it possible to achieve autologous transplantation. The first study for therapeutic application of iPS cells was reported with the mouse model of sickle-cell anemia, a blood disease caused by a defect in the β-globin gene (Hanna et al. 2007). They established iPS cells from the diseased mouse, and then repaired their genetic defects by homologous recombination. The injection of hematopoietic progenitors derived from genetically-corrected iPS cells could cure the diseased donor mouse. Another report showed the injection of iPS-derived cells directly into the liver of irradiated hemophilia A mice to improve their phenotypes (Xu et al. 2009). These are examples of ideal models for regenerative medicine. In human case, the idea of iPS cell therapy was also brought up by Raya and colleagues (Raya et al. 2009). At first, they corrected genes by introducing a lentiviral vector encoding *FANCA* or *FANCD2* in keratinocytes derived from Fonconi anemia patients. iPS cells derived from the patients with genetic correction can be differentiated into the hematopoietic lineage.

Moreover, dopaminergic neurons differentiated from iPS cells were able to improve the behavior of a Parkinson's disease model rat through transplantation into the brain (Wernig et al. 2008c). On the other hand, however, they found that residual undifferentiated iPS cells included in the cells for transplantation caused a teratoma. Undifferentiated cells and the subsequent tumor formation after transplantation remain to be a major complication of pluripotent cell therapies, not only with iPS cells, but also with ES cells.

The depletion of SSEA-1 positive cells, which means undifferentiated cells, from the cultures by fluorescence-activated cell sorter (FACS) can reduce the risk of teratoma formation after cell transplantation (Wernig et al. 2008c). This issue was analyzed with a different point of view in the work of Miura and colleagues (Miura et al. 2009). They differentiated 36 mouse iPS cell lines derived from embryonic or adult cells as well as ES cells into neural progenitor cells using the sphere culture method (Miura et al. 2009). They found that residual undifferentiated cells existed in less than 0.5% of ES cells or MEF-derived iPS cell lines. In contrast, TTF-derived iPS cell lines reproducibly showed a significantly higher ratio of undifferentiated cells. Interestingly, such differences did not provide clear relationship with the presence or absence of Myc transgenes.

Clinical trials using human ES cells have been carried out in the US, Korea, and UK. According to the preliminary report published in 2012, retinal pigment epithelium derived from human ES cells were transplanted into patients with Stargardt's macular dystrophy and dry age-related macular degeneration (Schwartz et al. 2012). The transplanted cells showed no signs of hyperproliferation, tumorigenicity, ectopic tissue formation, or apparent rejection after 4 months. Moreover vision seemed to improve in one patient. The ES cells studies would encourage future iPS cell trials.

8.7 Conclusion and Perspective

Human iPS cells have another potential utility as a tool for drug discovery or understanding the pathogenesis of diseases. Over the last couple of years, iPS cells were established from somatic cells of patients with refractory diseases such as Amyotrophic lateral sclerosis (ALS), Duchenne muscular dystrophy, Down syndrome and Parkinson's disease, familial dysautonomia, type I diabetes, beta-thalassemia, Rett syndrome, LEOPARD syndrome and Fanconi anemia (Park et al. 2008a; Maehr et al. 2009; Lee et al. 2009; Ye et al. 2009; Soldner et al. 2009; Raya et al. 2009; Carvajal-Vergara et al. 2010; Hotta et al. 2009). Heretofore, it was difficult to analyze the pathogenesis of specific diseases *in vitro* because large amount of the patient's cells are needed. iPS cells established from the patients may overcome the issue with their indefinite self-renewal ability. Combining the proliferation of undifferentiated iPS cells and the differentiation of specific cell types can yield an enormous amount of cells carrying the causal factors of diseases as associated with genetic mutations can be obtained, and used for large-scale screening or analyses.

If pathological conditions can be recapitulated *in vitro* using iPS cells from the patients, they could be used for screening therapeutically-effective molecules, and/or for the evaluation of medicine side-effects for each individual patient. For example, Long-QT syndromes (LQTS) are congenital or acquired types of diseases with delayed repolarization and subsequent depolarization of the heart. This can lead to a risk of ventricular fibrillation and unexpected death. A mutation in one of several disease-causing genes can bring on LQTS, and the effective medication is different among each type of LQTS. An epinephrine-loading test seems to be effective to determine if a patient has LQTS. However, this test may carry a certain degree of risk. Functional cardiomyocytes derived from patient's iPS cells can be used for the *in vitro* diagnosis of LQTS, thus reducing the burden and risk for the patient.

However, recapitulation pathogenesis with disease-specific iPS cells does not always work well. Dimos et al. established iPS cells derived from an ALS patient (Dimos et al. 2008). They successfully differentiated ALS-iPS cells into both glial cells and motor neurons. However, although the glial cells of ALS patient produce toxins and result in destroying motor neurons, they failed to show the phenomenon *in vitro* with iPS cells. In contrast, Ebert and colleagues generated iPS cells carrying spinal muscular atrophy (SMA) which is a neurological disorder developed during infancy and causes death. Motor neurons derived from SMA patient's iPS cells

showed a defect in the maturation of motor neurons (Ebert et al. 2009). Another paper also reported the success of reproducing a pathological condition. They choose Familial dysautonomia (FD) as a model of pathogenesis, which shows peripheral neuropathy induced by a reduction in the expression of the *IKBKAP* gene. They developed neural crest precursor cells from FD-iPS cells. As result, they observed a defect of cell migration and spontaneous differentiation into TuJ1 or ASCL1 positive neurons. In addition, they treated the cells with kinetin which could correct the expression of *IKBKAP* in mutant cells, and it markedly improved the phenotypes required for cell migration. Therefore, although not in all cases, these data indicate that the recapitulation of the pathogenesis and subsequent tests for drug effects are feasible.

At this time, the overriding issue remains the quality of iPS cells. It is not always true that integration-free and/or virus-free methods are the best methods for clinical application because insufficient reprogramming carries higher risks than transgenes. A direct comparison of iPS cells established with various techniques must be conducted by the same experimenter with a set of evaluation standards. Of course, human ES cells must be the benchmark for the evaluation of iPS cell quality. However, many differences are found among human ES cell clones with regard to differentiation potentials, gene expression patterns and the status of X-chromosome because of a variety of races (International Stem Cell Initiative 2007). This issue also applies to iPS cells. In addition, not only genetic backgrounds, but also the age of donors, cell types as sources of iPS cells and freshness such as passage number will be different in individual cases. The effects of these factors on the characteristics of iPS cells should therefore also be further investigated. One advantage of iPS cells is the potential usage of autologous and HLA-matched transplantation. A study raised question about this point as Zhao et al. showed immunogenicity of mouse iPS cells (Zhao et al. 2011). However they used undifferentiated iPS cells for transplantation which would never happen in medical transplantation. Nevertheless, immunogenicity of iPS cells should be carefully examined.

The continuing rapid progress in iPS cell research, as well as in direct reprogramming, is therefore expected to elucidate over time various new treatment strategies for intractable diseases.

Acknowledgement We'd like to thank the members of the Yamanaka laboratory and CiRA for their valuable support and discussions.

References

Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, Vassena R, Bili J, Pekarik V, Tiscornia G, Edel M, Boué S, Izpisúa Belmonte JC (2008) Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol 26(11):1276–1284

Ambrosetti DC, Basilico C, Dailey L (1997) Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. Mol Cell Biol 17(11):6321–6329

Aoi T, Yae K, Nakagawa M, Ichisaka T, Okita K, Takahashi K, Chiba T, Yamanaka S (2008) Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science 321(5889):699–702

Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R (2003) Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 17(1): 126–140

Banito A, Rashid ST, Acosta JC, Li S, Pereira CF, Geti I, Pinho S, Silva JC, Azuara V, Walsh M, Vallier L, Gil J (2009) Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev 23(18):2134–2139

Bar-Nur O, Russ HA, Efrat S, Benvenisty N (2011) Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. Cell Stem Cell 9(1):17–23

Ben-Nun IF, Montague SC, Houck ML, Tran HT, Garitaonandia I, Leonardo TR, Wang YC, Charter SJ, Laurent LC, Ryder OA, Loring JF (2011) Induced pluripotent stem cells from highly endangered species. Nat Methods 8(10):829–831

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125(2):315–326

Boiani M, Schöler HR (2005) Regulatory networks in embryo-derived pluripotent stem cells. Nat Rev Mol Cell Biol 6(11):872–884

Boland MJ, Hazen JL, Nazor KL, Rodriguez AR, Gifford W, Martin G, Kupriyanov S, Baldwin KK (2009) Adult mice generated from induced pluripotent stem cells. Nature 461(7260):91–94

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122(6):947–956

Burdon T, Stracey C, Chambers I, Nichols J, Smith A (1999) Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. Dev Biol 210(1):30–43

Carey BW, Markoulaki S, Hanna JH, Faddah DA, Buganim Y, Kim J, Ganz K, Steine EJ, Cassady JP, Creyghton MP, Welstead GG, Gao Q, Jaenisch R (2011) Reprogramming factor stoichiometry influences the epigenetic state and biological properties of induced pluripotent stem cells. Cell Stem Cell 9(6):588–598

Cartwright P, McLean C, Sheppard A, Rivett D, Jones K, Dalton S (2005) LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development 132(5):885–896

Carvajal-Vergara X, Sevilla A, D'Souza SL, Ang YS, Schaniel C, Lee DF, Yang L, Kaplan AD, Adler ED, Rozov R, Ge Y, Cohen N, Edelmann LJ, Chang B, Waghray A, Su J, Pardo S, Lichtenbelt KD, Tartaglia M, Gelb BD, Lemischka IR (2010) Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome. Nature 465(7299):808–812

Chambers I, Smith A (2004) Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene 23(43):7150–7160

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113(5):643–655

Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L (2009) Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol 27(3):275–280

Chen AE, Egli D, Niakan K, Deng J, Akutsu H, Yamaki M, Cowan C, Fitz-Gerald C, Zhang K, Melton DA, Eggan K (2009) Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. Cell Stem Cell 4(2):103–106

Cheng AM, Saxton TM, Sakai R, Kulkarni S, Mbamalu G, Vogel W, Tortorice CG, Cardiff RD, Cross JC, Muller WJ, Pawson T (1998) Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. Cell 95(6):793–803

Chew JL, Loh YH, Zhang W, Chen X, Tam WL, Yeap LS, Li P, Ang YS, Lim B, Robson P, Ng HH (2005) Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. Mol Cell Biol 25(14):6031–6046

Chin MH, Mason MJ, Xie W, Volinia S, Singer M, Peterson C, Ambartsumyan G, Aimiuwu O, Richter L, Zhang J, Khvorostov I, Ott V, Grunstein M, Lavon N, Benvenisty N, Croce CM, Clark AT, Baxter T, Pyle AD, Teitell MA, Pelegrini M, Plath K, Lowry WE (2009) Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. Cell Stem Cell 5(1):111–123

Cowan CA, Atienza J, Melton DA, Eggan K (2005) Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 309(5739):1369–1373

Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, Croft GF, Saphier G, Leibel R, Goland R, Wichterle H, Henderson CE, Eggan K (2008) Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science 321(5893):1218–1221

Doetschman T, Gregg RG, Maeda N, Hooper ML, Melton DW, Thompson S, Smithies O (1987) Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. Nature 330(6148): 576–578

Ebert AD, Yu J, Rose FF Jr, Mattis VB, Lorson CL, Thomson JA, Svendsen CN (2009) Induced pluripotent stem cells from a spinal muscular atrophy patient. Nature 457(7227):277–280

Eminli S, Utikal J, Arnold K, Jaenisch R, Hochedlinger K (2008) Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression. Stem Cells 26(10):2467–2474

Eminli S, Foudi A, Stadtfeld M, Maherali N, Ahfeldt T, Mostoslavsky G, Hock H, Hochedlinger K (2009) Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. Nat Genet 41(9):968–976

Esteban MA, Xu J, Yang J, Peng M, Qin D, Li W, Jiang Z, Chen J, Deng K, Zhong M, Cai J, Lai L, Pei D (2009) Generation of induced pluripotent stem cell lines from Tibetan miniature pig. J Biol Chem 284(26):17634–17640

Feng B, Jiang J, Kraus P, Ng JH, Heng JC, Chan YS, Yaw LP, Zhang W, Loh YH, Han J, Vega VB, Cacheux-Rataboul V, Lim B, Lufkin T, Ng HH (2009) Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. Nat Cell Biol 11(2):197–203

Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M (2009) Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci 85(8):348–362

Gai H, Leung EL, Costantino PD, Aguila JR, Nguyen DM, Fink LM, Ward DC, Ma Y (2009) Generation and characterization of functional cardiomyocytes using induced pluripotent stem cells derived from human fibroblasts. Cell Biol Int 33:1184–1193 [Epub ahead of print]

Giorgetti A, Montserrat N, Aasen T, Gonzalez F, Rodríguez-Pizà I, Vassena R, Raya A, Boué S, Barrero MJ, Corbella BA, Torrabadella M, Veiga A, Izpisua Belmonte JC (2009) Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2. Cell Stem Cell 5(4):353–357

Gurdon JB, Elsdale TR, Fischberg M (1958) Sexually mature individuals of Xenopus laevis from the transplantation of single somatic nuclei. Nature 182(4627):64–65

Haase A, Olmer R, Schwanke K, Wunderlich S, Merkert S, Hess C, Zweigerdt R, Gruh I, Meyer J, Wagner S, Maier LS, Han DW, Glage S, Miller K, Fischer P, Schöler HR, Martin U (2009) Generation of induced pluripotent stem cells from human cord blood. Cell Stem Cell 5(4): 434–441

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint BG, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302(5644):415–419

Hanna J, Wernig M, Markoulaki S, Sun CW, Meissner A, Cassady JP, Beard C, Brambrink T, Wu LC, Townes TM, Jaenisch R (2007) Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science 318(5858):1920–1923

Hanna J, Markoulaki S, Schorderet P, Carey BW, Beard C, Wernig M, Creyghton MP, Steine EJ, Cassady JP, Foreman R, Lengner CJ, Dausman JA, Jaenisch R (2008) Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell 18:133(2):250–264; Stem Cells 26(10):2467–2474

Honda A, Hirose M, Hatori M, Matoba S, Miyoshi H, Inoue K, Ogura A (2010) Generation of induced pluripotent stem cells in rabbits: potential experimental models for human regenerative medicine. J Biol Chem 285(41):31362–31369

Hong H, Takahashi K, Ichisaka T, Aoi T, Kanagawa O, Nakagawa M, Okita K, Yamanaka S (2009) Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature 460(7259):1132–1135

Hotta A, Cheung AY, Farra N, Vijayaragavan K, Séguin CA, Draper JS, Pasceri P, Maksakova IA, Mager DL, Rossant J, Bhatia M, Ellis J (2009) Isolation of human iPS cells using EOS lentiviral vectors to select for pluripotency. Nat Methods 6(5):370–376

Huangfu D, Maehr R, Guo W, Eijkelenboom A, Snitow M, Chen AE, Melton DA (2008a) Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nat Biotechnol 26(7):795–797

Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, Chen S, Muhlestein W, Melton DA (2008b) Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol 26(11):1269–1275

International Stem Cell Initiative, Adewumi O, Aflatoonian B, Ahrlund-Richter L, Amit M, Andrews PW, Beighton G, Bello PA, Benvenisty N, Berry LS, Bevan S, Blum B, Brooking J, Chen KG, Choo AB, Churchill GA, Corbel M, Damjanov I, Draper JS, Dvorak P, Emanuelsson K, Fleck RA, Ford A, Gertow K, Gertsenstein M, Gokhale PJ, Hamilton RS, Hampl A, Healy LE, Hovatta O, Hyllner J, Imreh MP, Itskovitz-Eldor J, Jackson J, Johnson JL, Jones M, Kee K, King BL, Knowles BB, Lako M, Lebrin F, Mallon BS, Manning D, Mayshar Y, McKay RD, Michalska AE, Mikkola M, Mileikovsky M, Minger SL, Moore HD, Mummery CL, Nagy A, Nakatsuji N, O'Brien CM, Oh SK, Olsson C, Otonkoski T, Park KY, Passier R, Patel H, Patel M, Pedersen R, Pera MF, Piekarczyk MS, Pera RA, Reubinoff BE, Robins AJ, Rossant J, Rugg-Gunn P, Schulz TC, Semb H, Sherrer ES, Siemen H, Stacey GN, Stojkovic M, Suemori H, Szatkiewicz J, Turetsky T, Tuuri T, van den Brink S, Vintersten K, Vuoristo S, Ward D, Weaver TA, Young LA, Zhang W (2007) Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol 25(7):803–816

Judson RL, Babiarz JE, Venere M, Blelloch R (2009) Embryonic stem cell-specific microRNAs promote induced pluripotency. Nat Biotechnol 27(5):459–461

Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K (2009) Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature 458(7239):771–775

Kamachi Y, Uchikawa M, Kondoh H (2000) Pairing SOX off: with partners in the regulation of embryonic development. Trends Genet 16(4):182–187

Kang L, Wang J, Zhang Y, Kou Z, Gao S (2009) iPS cells can support full-term development of tetraploid blastocyst-complemented embryos. Cell Stem Cell 5(2):135–138

Karumbayaram S, Novitch BG, Patterson M, Umbach JA, Richter L, Lindgren A, Conway AE, Clark AT, Goldman SA, Plath K, Wiedau-Pazos M, Kornblum HI, Lowry WE (2009) Directed differentiation of human-induced pluripotent stem cells generates active motor neurons. Stem Cells 27(4):806–811

Kawai J, Shinagawa A, Shibata K, Yoshino M, Itoh M, Ishii Y, Arakawa T, Hara A, Fukunishi Y, Konno H, Adachi J, Fukuda S, Aizawa K, Izawa M, Nishi K, Kiyosawa H, Kondo S, Yamanaka I, Saito T, Okazaki Y, Gojobori T, Bono H, Kasukawa T, Saito R, Kadota K, Matsuda H, Ashburner M, Batalov S, Casavant T, Fleischmann W, Gaasterland T, Gissi C, King B, Kochiwa H, Kuehl P, Lewis S, Matsuo Y, Nikaido I, Pesole G, Quackenbush J, Schriml LM, Staubli F, Suzuki R, Tomita M, Wagner L, Washio T, Sakai K, Okido T, Furuno M, Aono H, Baldarelli R, Barsh G, Blake J, Boffelli D, Bojunga N, Carninci P, de Bonaldo MF, Brownstein MJ, Bult C, Fletcher C, Fujita M, Gariboldi M, Gustincich S, Hill D, Hofmann M, Hume DA, Kamiya M, Lee NH, Lyons P, Marchionni L, Mashima J, Mazzarelli J, Mombaerts P, Nordone P, Ring B, Ringwald M, Rodriguez I, Sakamoto N, Sasaki H, Sato K, Schönbach C, Seya T, Shibata Y,

Storch KF, Suzuki H, Toyo-oka K, Wang KH, Weitz C, Whittaker C, Wilming L, Wynshaw-Boris A, Yoshida K, Hasegawa Y, Kawaji H, Kohtsuki S, Hayashizaki Y, RIKEN Genome Exploration Research Group Phase II Team and the FANTOM Consortium (2001) Functional annotation of a full-length mouse cDNA collection. Nature 409(6821):685–690

Kawamura T, Suzuki J, Wang YV, Menendez S, Morera LB, Raya A, Wahl GM, Belmonte JC (2009) Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature 460(7259):1140–1144

Kim D, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, Ko S, Yang E, Cha KY, Lanza R, Kim KS (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell 4(6):472–476

Kim JB, Zaehres H, Wu G, Gentile L, Ko K, Sebastiano V, Araúzo-Bravo MJ, Ruau D, Han DW, Zenke M, Schöler HR (2008) Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 454(7204):646–650

Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, Kim J, Aryee MJ, Ji H, Ehrlich LI, Yabuuchi A, Takeuchi A, Cunniff KC, Hongguang H, McKinney-Freeman S, Naveiras O, Yoon TJ, Irizarry RA, Jung N, Seita J, Hanna J, Murakami P, Jaenisch R, Weissleder R, Orkin SH, Weissman IL, Feinberg AP, Daley GQ (2010) Epigenetic memory in induced pluripotent stem cells. Nature 467(7313):285–290

Lee G, Papapetrou EP, Kim H, Chambers SM, Tomishima MJ, Fasano CA, Ganat YM, Menon J, Shimizu F, Viale A, Tabar V, Sadelain M, Studer L (2009) Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. Nature 461(7262):402–406

Li H, Collado M, Villasante A, Strati K, Ortega S, Cañamero M, Blasco MA, Serrano M (2009) The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature 460(7259):1136–1139

Li Y, Cang M, Lee AS, Zhang K, Liu D (2011) Reprogramming of sheep fibroblasts into pluripotency under a drug-inducible expression of mouse-derived defined factors. PLoS One 6(1):e15947

Liao J, Wu Z, Wang Y, Cheng L, Cui C, Gao Y, Chen T, Rao L, Chen S, Jia N, Dai H, Xin S, Kang J, Pei G, Xiao L (2008) Enhanced efficiency of generating induced pluripotent stem (iPS) cells from human somatic cells by a combination of six transcription factors. Cell Res 18(5):600–603

Liao J, Cui C, Chen S, Ren J, Chen J, Gao Y, Li H, Jia N, Cheng L, Xiao H, Xiao L (2009) Generation of induced pluripotent stem cell lines from adult rat cells. Cell Stem Cell 4(1):11–15

Lister R, Pelizzola M, Kida YS, Hawkins RD, Nery JR, Hon G, Antosiewicz-Bourget J, O'Malley R, Castanon R, Klugman S, Downes M, Yu R, Stewart R, Ren B, Thomson JA, Evans RM, Ecker JR (2011) Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature 471(7336):68–73

Liu H, Zhu F, Yong J, Zhang P, Hou P, Li H, Jiang W, Cai J, Liu M, Cui K, Qu X, Xiang T, Lu D, Chi X, Gao G, Ji W, Ding M, Deng H (2008) Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. Cell Stem Cell 3(6):587–590

Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38(4):431–440

Loh YH, Agarwal S, Park IH, Urbach A, Huo H, Heffner GC, Kim K, Miller JD, Ng K, Daley GQ (2009) Generation of induced pluripotent stem cells from human blood. Blood 113(22): 5476–5479

Lowry WE, Richter L, Yachechko R, Pyle AD, Tchieu J, Sridharan R, Clark AT, Plath K (2008) Generation of human induced pluripotent stem cells from dermal fibroblasts. Proc Natl Acad Sci USA 105(8):2883–2888

Lyssiotis CA, Foreman RK, Staerk J, Garcia M, Mathur D, Markoulaki S, Hanna J, Lairson LL, Charette BD, Bouchez LC, Bollong M, Kunick C, Brinker A, Cho CY, Schultz PG, Jaenisch R (2009) Reprogramming of murine fibroblasts to induced pluripotent stem cells with chemical complementation of Klf4. Proc Natl Acad Sci USA 106(22):8912–8917

Maehr R, Chen S, Snitow M, Ludwig T, Yagasaki L, Goland R, Leibel RL, Melton DA (2009) Generation of pluripotent stem cells from patients with type 1 diabetes. Proc Natl Acad Sci USA 106:15768–15773

Maekawa M, Yamaguchi K, Nakamura T, Shibukawa R, Kodanaka I, Ichisaka T, Kawamura Y, Mochizuki H, Goshima N, Yamanaka S (2011) Direct reprogramming of somatic cells is promoted by maternal transcription factor Glis1. Nature 474(7350):225–229

Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yachechko R, Tchieu J, Jaenisch R, Plath K, Hochedlinger K (2007) Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell 1(1):55–70

Mali P, Ye Z, Hommond HH, Yu X, Lin J, Chen G, Zou J, Cheng L (2008) Improved efficiency and pace of generating induced pluripotent stem cells from human adult and fetal fibroblasts. Stem Cells 26(8):1998–2005

Marión RM, Strati K, Li H, Murga M, Blanco R, Ortega S, Fernandez-Capetillo O, Serrano M, Blasco MA (2009) A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. Nature 460(7259):1149–1153

Marson A, Foreman R, Chevalier B, Bilodeau S, Kahn M, Young RA, Jaenisch R (2008) Wnt signaling promotes reprogramming of somatic cells to pluripotency. Cell Stem Cell 3(2):132–135

Masui S, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, Okochi H, Okuda A, Matoba R, Sharov AA, Ko MS, Niwa H (2007) Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol 9(6):625–635

Matsui T, Leung D, Miyashita H, Maksakova IA, Miyachi H, Kimura H, Tachibana M, Lorincz MC, Shinkai Y (2010) Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. Nature 464(7290):927–931

Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature 454(7205):766–770

Mikkelsen TS, Hanna J, Zhang X, Ku M, Wernig M, Schorderet P, Bernstein BE, Jaenisch R, Lander ES, Meissner A (2008) Dissecting direct reprogramming through integrative genomic analysis. Nature 454(7200):49–55

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113(5):631–642

Miura K, Okada Y, Aoi T, Okada A, Takahashi K, Okita K, Nakagawa M, Koyanagi M, Tanabe K, Ohnuki M, Ogawa D, Ikeda E, Okano H, Yamanaka S (2009) Variation in the safety of induced pluripotent stem cell lines. Nat Biotechnol 27(8):743–745

Miyoshi N, Ishii H, Nagano H, Haraguchi N, Dewi DL, Kano Y, Nishikawa S, Tanemura M, Mimori K, Tanaka F, Saito T, Nishimura J, Takemasa I, Mizushima T, Ikeda M, Yamamoto H, Sekimoto M, Doki Y, Mori M (2011) Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell 8(6):633–638

Nagy K, Sung HK, Zhang P, Laflamme S, Vincent P, Agha-Mohammadi S, Woltjen K, Monetti C, Michael IP, Smith LC, Nagy A (2011) Induced pluripotent stem cell lines derived from equine fibroblasts. Stem Cell Rev 7(3):693–702

Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 26(1):101–106

Narazaki G, Uosaki H, Teranishi M, Okita K, Kim B, Matsuoka S, Yamanaka S, Yamashita JK (2008) Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. Circulation 118(5):498–506

Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Schöler H, Smith A (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95(3):379–391

Nishimoto M, Fukushima A, Okuda A, Muramatsu M (1999) The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. Mol Cell Biol 19(8):5453–5465

Nishimura K, Sano M, Ohtaka M, Furuta B, Umemura Y, Nakajima Y, Ikehara Y, Kobayashi T, Segawa H, Takayasu S, Sato H, Motomura K, Uchida E, Kanayasu-Toyoda T, Asashima M, Nakauchi H, Yamaguchi T, Nakanishi M (2011) Development of defective and persistent

Sendai virus vector: a unique gene delivery/expression system ideal for cell reprogramming. J Biol Chem 286(6):4760–4771
Niwa H (2007) How is pluripotency determined and maintained? Development 134(4):635–646
Niwa H, Burdon T, Chambers I, Smith A (1998) Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev 12(13):2048–2060
Niwa H, Miyazaki J, Smith AG (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 24(4):372–376
Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, Yagi R, Rossant J (2005) Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell 123(5):917–929
Okamoto K, Okazawa H, Okuda A, Sakai M, Muramatsu M, Hamada H (1990) A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell 60(3):461–472
Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448(7151):313–317
Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S (2008) Generation of mouse induced pluripotent stem cells without viral vectors. Science 322(5903):949–953
Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, Hong H, Nakagawa M, Tanabe K, Tezuka K, Shibata T, Kunisada T, Takahashi M, Takahashi J, Saji H, Yamanaka S (2011) A more efficient method to generate integration-free human iPS cells. Nat Methods 8(5):409–412
Osakada F, Jin ZB, Hirami Y, Ikeda H, Danjyo T, Watanabe K, Sasai Y, Takahashi M (2009) In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. J Cell Sci 122(Pt 17):3169–3179
Papapetrou EP, Tomishima MJ, Chambers SM, Mica Y, Reed E, Menon J, Tabar V, Mo Q, Studer L, Sadelain M (2009) Stoichiometric and temporal requirements of Oct4, Sox2, Klf4, and c-Myc expression for efficient human iPSC induction and differentiation. Proc Natl Acad Sci USA 106(31):12759–12764
Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, Lensch MW, Cowan C, Hochedlinger K, Daley GQ (2008a) Disease-specific induced pluripotent stem cells. Cell 134(5):877–886
Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ (2008b) Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451(7175):141–146
Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA (2002) "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 298(5593):597–600
Raya A, Rodríguez-Pizà I, Guenechea G, Vassena R, Navarro S, Barrero MJ, Consiglio A, Castellà M, Río P, Sleep E, González F, Tiscornia G, Garreta E, Aasen T, Veiga A, Verma IM, Surrallés J, Bueren J, Izpisúa Belmonte JC (2009) Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature 460(7251):53–59
Reményi A, Lins K, Nissen LJ, Reinbold R, Schöler HR, Wilmanns M (2003) Crystal structure of a POU/HMG/DNA ternary complex suggests differential assembly of Oct4 and Sox2 on two enhancers. Genes Dev 17(16):2048–2059
Robertson E, Bradley A, Kuehn M, Evans M (1986) Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323(6087):445–448
Schenke-Layland K, Rhodes KE, Angelis E, Butylkova Y, Heydarkhan-Hagvall S, Gekas C, Zhang R, Goldhaber JI, Mikkola HK, Plath K, MacLellan WR (2008) Reprogrammed mouse fibroblasts differentiate into cells of the cardiovascular and hematopoietic lineages. Stem Cells 26(6):1537–1546
Schöler HR, Ruppert S, Suzuki N, Chowdhury K, Gruss P (1990) New type of POU domain in germ line-specific protein Oct-4. Nature 344(6265):435–439
Schwartz SD, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, Mickunas E, Gay R, Klimanskaya I, Lanza R (2012) Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet 379(9817):713–720
Seki T, Yuasa S, Oda M, Egashira T, Yae K, Kusumoto D, Nakata H, Tohyama S, Hashimoto H, Kodaira M, Okada Y, Seimiya H, Fusaki N, Hasegawa M, Fukuda K (2010) Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. Cell Stem Cell 7(1):11–14

Senju S, Haruta M, Matsunaga Y, Fukushima S, Ikeda T, Takahashi K, Okita K, Yamanaka S, Nishimura Y (2009) Characterization of dendritic cells and macrophages generated by directed differentiation from mouse induced pluripotent stem cells. Stem Cells 27(5):1021–1031

Shi Y, Desponts C, Do JT, Hahm HS, Schöler HR, Ding S (2008) Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. Cell Stem Cell 3(5):568–574

Shimada H, Nakada A, Hashimoto Y, Shigeno K, Shionoya Y, Nakamura T (2010) Generation of canine induced pluripotent stem cells by retroviral transduction and chemical inhibitors. Mol Reprod Dev 77(1):2

Silva J, Barrandon O, Nichols J, Kawaguchi J, Theunissen TW, Smith A (2008) Promotion of reprogramming to ground state pluripotency by signal inhibition. PLoS Biol 6(10):e253

Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, Hargus G, Blak A, Cooper O, Mitalipova M, Isacson O, Jaenisch R (2009) Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell 136(5):964–977

Song Z, Cai J, Liu Y, Zhao D, Yong J, Duo S, Song X, Guo Y, Zhao Y, Qin H, Yin X, Wu C, Che J, Lu S, Ding M, Deng H (2009) Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. Cell Res 19:1233–1242. [Epub ahead of print]

Stadtfeld M, Brennand K, Hochedlinger K (2008a) Reprogramming of pancreatic beta cells into induced pluripotent stem cells. Curr Biol 18(12):890–894

Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K (2008b) Induced pluripotent stem cells generated without viral integration. Science 322(5903):945–949

Sumer H, Liu J, Malaver-Ortega LF, Lim ML, Khodadadi K, Verma PJ (2011) NANOG is a key factor for induction of pluripotency in bovine adult fibroblasts. J Anim Sci 89(9):2708–2716

Tada M, Takahama Y, Abe K, Nakatsuji N, Tada T (2001) Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr Biol 11(19):1553–1558

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676

Takahashi K, Mitsui K, Yamanaka S (2003) Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. Nature 423(6939):541–545

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872

Takayama N, Nishimura S, Nakamura S, Shimizu T, Ohnishi R, Endo H, Yamaguchi T, Otsu M, Nishimura K, Nakanishi M, Sawaguchi A, Nagai R, Takahashi K, Yamanaka S, Nakauchi H, Eto K (2010) Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells. J Exp Med 207(13):2817–2830

Tashiro K, Inamura M, Kawabata K, Sakurai F, Yamanishi K, Hayakawa T, Mizuguchi H (2009) Efficient adipocyte and osteoblast differentiation from mouse induced pluripotent stem cells by adenoviral transduction. Stem Cells 27(8):1802–1811

Taura D, Sone M, Homma K, Oyamada N, Takahashi K, Tamura N, Yamanaka S, Nakao K (2009) Induction and isolation of vascular cells from human induced pluripotent stem cells. Arterioscler Thromb Vasc Biol 29(7):1100–1103

Thomas KR, Capecchi MR (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51(3):503–512

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282(5391):1145–1147

Tokuzawa Y, Kaiho E, Maruyama M, Takahashi K, Mitsui K, Maeda M, Niwa H, Yamanaka S (2003) Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. Mol Cell Biol 23(8):2699–2708

Tsubooka N, Ichisaka T, Okita K, Takahashi K, Nakagawa M, Yamanaka S (2009) Roles of Sall4 in the generation of pluripotent stem cells from blastocysts and fibroblasts. Genes Cells 14(6):683–694

Utikal J, Polo JM, Stadtfeld M, Maherali N, Kulalert W, Walsh RM, Khalil A, Rheinwald JG, Hochedlinger K (2009) Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. Nature 460(7259):1145–1148

Viczian AS, Solessio EC, Lyou Y, Zuber ME (2009) Generation of functional eyes from pluripotent cells. PLoS Biol 7(8):e1000174

Wang W, Yang J, Liu H, Lu D, Chen X, Zenonos Z, Campos LS, Rad R, Guo G, Zhang S, Bradley A, Liu P (2011) Rapid and efficient reprogramming of somatic cells to induced pluripotent stem cells by retinoic acid receptor gamma and liver receptor homolog 1. Proc Natl Acad Sci USA 108(45):18283–18288

Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ, Cowan C, Schlaeger TM, Rossi DJ (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 7(5):618–630

Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448(7151):318–324

Wernig M, Lengner CJ, Hanna J, Lodato MA, Steine E, Foreman R, Staerk J, Markoulaki S, Jaenisch R (2008a) A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. Nat Biotechnol 26(8):916–924

Wernig M, Meissner A, Cassady JP, Jaenisch R (2008b) c-Myc is dispensable for direct reprogramming of mouse fibroblasts. Cell Stem Cell 2(1):10–12

Wernig M, Zhao JP, Pruszak J, Hedlund E, Fu D, Soldner F, Broccoli V, Constantine-Paton M, Isacson O, Jaenisch R (2008c) Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. Proc Natl Acad Sci USA 105(15):5856–5861

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385(6619):810–813

Wolf D, Goff SP (2007) TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. Cell 131(1):46–57

Wolf D, Goff SP (2009) Embryonic stem cells use ZFP809 to silence retroviral DNAs. Nature 458(7242):1201–1204

Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hämäläinen R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung HK, Nagy A (2009) piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 458(7239):766–770

Xu D, Alipio Z, Fink LM, Adcock DM, Yang J, Ward DC, Ma Y (2009) Phenotypic correction of murine hemophilia A using an iPS cell-based therapy. Proc Natl Acad Sci USA 106(3):808–813

Yamanaka S (2009) A fresh look at iPS cells. Cell 137(1):13–17

Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT (2010) iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem Cells Dev 19(4):469–480

Ye L, Chang JC, Lin C, Sun X, Yu J, Kan YW (2009) Induced pluripotent stem cells offer new approach to therapy in thalassemia and sickle cell anemia and option in prenatal diagnosis in genetic diseases. Proc Natl Acad Sci USA 106(24):9826–9830

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318(5858):1917–1920

Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, Thomson JA (2009) Human induced pluripotent stem cells free of vector and transgene sequences. Science 324(5928):797–801

Yusa K, Rad R, Takeda J, Bradley A (2009) Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. Nat Methods 6(5):363–369

Zhang D, Jiang W, Liu M, Sui X, Yin X, Chen S, Shi Y, Deng H (2009a) Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. Cell Res 19(4):429–438

Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ (2009b) Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 104(4):e30–e41

Zhao Y, Yin X, Qin H, Zhu F, Liu H, Yang W, Zhang Q, Xiang C, Hou P, Song Z, Liu Y, Yong J, Zhang P, Cai J, Liu M, Li H, Li Y, Qu X, Cui K, Zhang W, Xiang T, Wu Y, Zhao Y, Liu C, Yu

C, Yuan K, Lou J, Ding M, Deng H (2008) Two supporting factors greatly improve the efficiency of human iPSC generation. Cell Stem Cell 3(5):475–479

Zhao XY, Li W, Lv Z, Liu L, Tong M, Hai T, Hao J, Guo CL, Ma QW, Wang L, Zeng F, Zhou Q (2009) iPS cells produce viable mice through tetraploid complementation. Nature 461(7260):86–90

Zhao T, Zhang ZN, Rong Z, Xu Y (2011) Immunogenicity of induced pluripotent stem cells. Nature 474(7350):212–215

Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Schöler HR, Duan L, Ding S (2009) Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4(5):381–384

Chapter 9
Spermatogonial Stem Cells

Ilya Chuykin, Michael Stauske, and Kaomei Guan

Abstract Spermatogonial stem cells (SSCs) constitute one of the most important stem cell systems in the adult body. SSCs are unipotent and respond for spermatogenesis in the male as they can only differentiate into sperms within the testicular niche. The long-term culture of SSCs without loss of their properties provides the opportunity to develop therapeutic strategies and re-initiate spermatogenesis for the patients who become infertile after cancer therapy. Moreover, SSCs can be spontaneously reprogrammed into pluripotent germline stem cells (GSCs) similar to embryonic stem cells (ESCs) when they are removed from their *in vivo* niche and cultured *in vitro* under specific conditions. The advantage of pluripotent GSCs over induced pluripotent stem cells is that conversion of SSCs into pluripotent GSCs does not require addition of genes using the virus system, which may avoid unpredictable genetic dysfunction. In addition, this may also circumvent ethical problems associated with human ESCs. The ability to generate patient-specific pluripotent GSCs for autologous transplantation provides the opportunity for cell replacement therapy without the need for immunosuppressant. In this review, we discuss the origin, properties and regenerative potential of SSCs. We summarize recent research findings regarding the mechanisms that regulate the self-renewal of SSCs. We believe that studying the biology of SSCs provides us important information to better understand male fertility. Furthermore, we address the contribution of SSCs and pluripotent GSCs to stem cell-based therapy for infertility treatment as well as for organ regeneration in the future.

I. Chuykin, Ph.D. • M. Stauske, M.Sc. • K. Guan, Ph.D. (✉)
Department of Cardiology and Pneumology, Georg-August-University of Göttingen,
Robert-Koch-Str. 40, 37075 Göttingen, Germany
e-mail: kguan@med.uni-goettingen.de

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_9,

9.1 Introduction

In sexually reproducing animals the function of germ cells is to bring the genetic information from one generation to the next. In females oocytes provide one part of the genetic information, whereas in males spermatozoa own the other part. After fertilization both haploid genomes come together and a new offspring is able to grow up. The unique capacity of germ cells to transmit information from parent to offspring has interested scientists for many years.

In the adult testis, the seminiferous epithelium is mainly composed of somatic cells and spermatogenic cells (Fig. 9.1a, c). Sertoli cells, the somatic cells in the seminiferous epithelium are crucial for the coordination of spermatogenic events, thereby contributing to the well-organized structural and functional construction of the seminiferous epithelium (Jegou 1992). Spermatogenic cells are made of several generations of dividing and differentiating cells, which are involved in the production of spermatozoa. Spermatogenic cells (spermatogonia, spermatocytes, and spermatids) are organized in layers. Spermatogonia reside at the basement membrane of

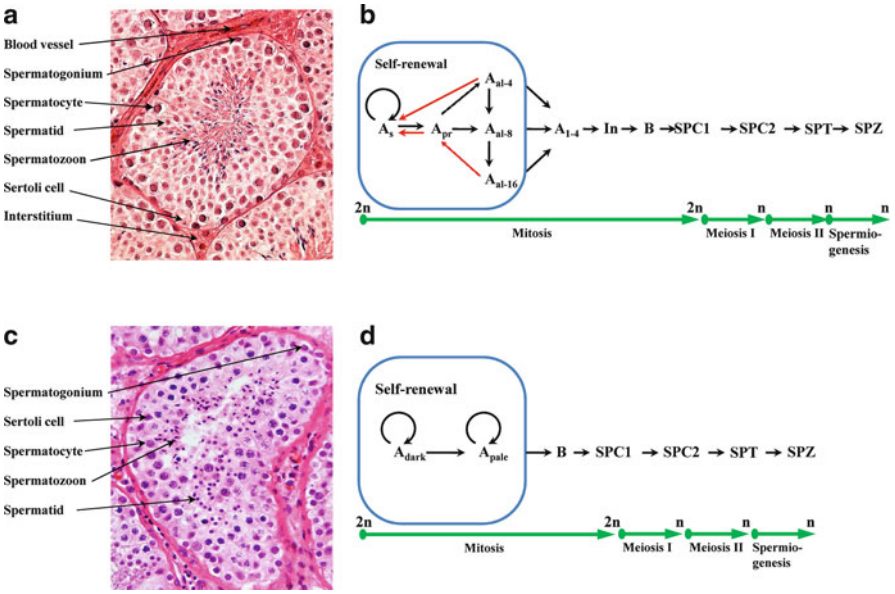


Fig. 9.1 Proposed models of spermatogonial subpopulations in mouse and human testes. (**a**) Cross section of mouse seminiferous tubule with spermatogonial subpopulations and somatic cells. (**b**) Model of self-renewal within the spermatogonial stem cell compartment and spermatogenesis in the mouse suggested by Nakagawa et al. 2010. According to this model spermatogonia type A_{pr} and A_{al} can dedifferentiate into SSCs (A_s spermatogonia). (**c**) Cross section of human seminiferous tubule with spermatogonial subpopulations and somatic cells. (**d**) Scheme for human spermatogonial stem cell renewal and spermatogenesis proposed by Clermont in the 1960s. A_s single type A spermatogonium, A_{pr} paired type A spermatogonium, A_{al} aligned type A spermatogonium, *In* intermediate spermatogonium, *SPC1* primary spermatocyte, *SPC2* secondary spermatocyte, *SPT* spermatid, *SPZ* spermatozoon

seminiferous tubules. Spermatocytes are generally located in the middle of the seminiferous epithelium and spermatids (round and elongated) are located in the adluminal region. Spermatogenesis is a well-organized and complex process, which starts with a small number of spermatogonial stem cells (SSCs), the male germline stem cells (GSCs), and can generate 100 million spermatozoa each day in adult males (de Rooij 1998). Spermatogenesis begins at 5–7 days after birth in rodents and 10–13 years after birth in men. The time from SSC differentiation to production of mature spermatozoa is about 35 days in the mouse and 64 days in the human (Brinster 2007). Spermatogenesis can be divided into three distinct phases: spermatogonial, spermatocyte and spermatid phases. In the spermatogonial phase (also known as mitotic phase or premeiosis), primary spermatocytes are generated as a result of proliferation and differentiation of spermatogonia. In the spermatocyte phase (meiosis I/II), each primary spermatocyte divides into two secondary spermatocytes during meiosis I, and each secondary spermatocyte into two spermatids during meiosis II. In the spermatid phase (also called as spermiogenesis), spermatozoa, also known as sperm cells are formed as a result of the metamorphosis of spermatids (Clermont 1972) (Fig. 9.1b, d). The timing of sequential steps in spermatogenesis is tightly regulated by genes of the germ cell, and Sertoli cells support the differentiation process.

SSCs, the undifferentiated spermatogonia constitute a small population of cells ($2–3 \times 10^4$ per adult mouse testis). Similar to other adult stem cells, SSCs have the capability to self-renew while remaining capable of generating numerous differentiated daughter cells. The small number of SSCs in the adult testis and the complexity of the microenvironment are the main difficulties in SSC research. Studies with transplantation in the adult mouse demonstrate the potential of SSCs in clinical application for the treatment of male infertility (Brinster and Zimmermann 1994; Kanatsu-Shinohara et al. 2006; Kubota et al. 2004b; Kubota and Brinster 2006). Previous studies show that using cryopreservation and SSC transplantation, cancer patients undergoing chemotherapy or radiotherapy can retain their fertility to safeguard their germline (Brinster 2007; Ryu et al. 2006).

Furthermore, recent research shows that under appropriate culture conditions, both neonatal and adult SSCs in the mouse are able to convert into pluripotent embryonic stem (ES)-like cells which can differentiate into derivatives of all three germ layers (Guan et al. 2006; Kanatsu-Shinohara et al. 2004). Similar results have also been reported with adult human SSCs (Conrad et al. 2008; He et al. 2010, 2012; Kossack et al. 2009). However, the pluripotent character of those human cells with respect to gene expression profile and ability to generate teratomas has been called into question (Ko et al. 2010; Tapia et al. 2011). The derivation of pluripotent stem cells from human testicular tissue may lead to a new source of autologous cells in regeneration of damaged organs. A thorough understanding of the molecular mechanisms, especially growth factors and signaling pathways, regulating the fate determination of SSCs has important implications for basic research and for their potential therapeutic application in patients.

In the present review, we focus on summarizing the origin and characteristics/properties of male GSCs, on understanding growth factors and signaling pathways

that regulate proliferation, differentiation, and fate decisions of SSCs, as well as on discussing their implications for basic research and for therapeutic application, with special focus on organ regeneration and infertility.

9.2 Derivation and Classification

9.2.1 Origin of SSCs

In many animal phyla, including insects, roundworms, and vertebrates, the distinction between somatic and germ cells occurs at the very early stage of embryonic development. All gametes arise from so-called primordial germ cells (PGCs), the embryonic precursors of the male and female germline. In insects and amphibians, there is a zone found in the cytoplasm of an egg cell, which contains determinants (RNA and protein components) that play an important role in PGC specification. This zone is called germ plasm or pole plasm. Only blastomeres incorporating the germ plasm develop into PGCs. The components of germ plasm prevent PGCs from differentiating into somatic cells by repression of the global transcriptional machinery (Strome and Lehmann 2007). Therefore, the germ plasm or pole plasm is often used as a convenient marker to trace the early ontogeny of germ cells in insects and amphibians (Eddy 1975; Eddy and Hahnel 1983; Mahowald and Hennen 1971).

In mammals such cytoplasmic determinants specifying PGCs are not detected. In early mammalian embryogenesis, the zygote divides three times resulting in a mass of eight cells having equal totipotency. At the 16-cell stage, the morula consists of a small group of internal cells, which remain pluripotent and give rise to the inner cell mass (ICM), and a larger group of external cells at the periphery, which become the trophectoderm cells, the first differentiated embryonic cell types. In the blastocyst, the ICM and the trophoblast cells become separate cell layers, neither of which contributes cells to the other group. Subsequently, a part of ICM differentiates into the primitive endoderm, and the remaining part of the ICM cells develops into the amniotic ectoderm and the embryonic epiblast. The embryonic epiblast is believed to be pluripotent and able to give rise to all cells of the three embryonic germ layers, as well as germ cells. PGCs are derived from the epiblast during gastrulation in rodents and humans (McLaren 2003).

In the mouse, the bone morphogenetic proteins (BMPs) produced by the extraembryonic ectoderm induce a small number of epiblast cells to become PGC precursors (Lawson et al. 1999). A group of about 50–100 cells are first distinguishable at embryonic day 7.25–7.5 within the extraembryonic mesoderm in the distal portion of the primitive streak and at the base of allantoic buds. In humans, at about 21–22 days of gestation PGCs are first recognizable at the same region as that in the mouse, the wall of yolk sac near the developing allantois (De Felici et al. 2004). Both mouse and human PGCs are recognized by their alkaline phosphatase activity (McLaren 2003). One reason that PGCs evolve outside the actual embryo in extraembryonic

tissues might be that here somatic paracrine factors cannot reach these cells and therefore they escape from a somatic cell fate. The precise molecular mechanism to establish the germline is not yet clear, but there are evidences pointing out an important role of transcription inhibitor B lymphocyte-induced maturation protein 1 (Blimp1, also Prdm1) (Ohinata et al. 2005). In addition, the expression of homeotic genes (*Hox*-genes) commonly present in somatic cells is downregulated in developing PGCs (Saitou et al. 2002). PGCs express germline-specific transcriptional factors and genes, such as *Oct4*, *Stella* (also known as *PGC7* and *Dppa3*), *Nanos3*, *Dead end*, *Blimp1* and *Vasa* (McLaren 2003; Saitou et al. 2002; Sato et al. 2002; Yabuta et al. 2006). After collecting at the allantois, the PGCs propagate and at the same time move from the adjacent yolk sac through the hind gut and dorsal mesentery into the genital ridges.

This process of division and migration of PGCs is strictly dependent on c-Kit/stem cell factor (SCF) signal transduction pathway. Mouse embryos homozygous for mutation in *c-Kit* gene (*W*) are deficient in germ cells (Buehr et al. 1993). In the absence of SCF, the c-Kit ligand, the motility of PGCs is dramatically decreased (Gu et al. 2009). The migration of PGCs is critically dependent on the interaction with extracellular matrix proteins, especially, with laminin (García-Castro et al. 1997), and cell surface receptor subunit β1-integrin plays an important role in colonization of the genital ridges, as in *β1-integrin* knockouts PGCs did not enter the embryonic gonads as efficiently as in wild type mice (Anderson et al. 1999). Initially PGCs proliferate after colonization and then enter the mitotic arrest in males (McLaren 2003).

At mouse embryonic day 12.5, the gonad of males becomes morphologically different from females. In human, the first signs of sexual differentiation appear at the end of week 7. In the male genital ridge, PGCs become enclosed by the somatic supporting cells, the differentiating Sertoli cells, and seminiferous cords are formed. The germ cells residing within seminiferous cords are called gonocytes and differ morphologically from PGCs. At embryonic day 13.5 in mice and at 18–20 weeks in humans, gonocytes arrest in the G0/G1 phase of the cell cycle and cease mitosis. The halt of proliferation is characteristic for the transition from PGCs to gonocytes, and the level of c-Kit receptor tyrosine kinase expression is decreased (Donovan and de Miguel 2007). Gonocytes lose expression of SSEA-1, which is expressed by PGCs and begin to express the germ cell nuclear antigen 1 (GCNA-1), an antigen of unknown function, recognized by a rat monoclonal antibody. The gonocytes also lose adhesiveness to fibronectin and laminin (De Felici and Dolci 1989; Donovan and de Miguel 2007), and at the same time increase in cell size more than fourfold. In the fetus, gonocytes are located in the center of the tubules. Following birth in mammals, gonocytes migrate to the seminiferous tubule basement membrane and reenter the cell cycle. At day 1 after birth in the mouse testis, the ^{3}H Thymidine labeling index of gonocytes is 10.4%, 20.1% at day 2, and 24.1% at day 3 (Donovan and de Miguel 2007; Vergouwen et al. 1991), at which time the first A spermatogonia (also known as SSCs) are identified (de Rooij 1998; Spradling et al. 2001). In mice, the SSC pool arises from gonocytes approximately 6 days after birth.

From the puberty on the task of SSCs in the male is to provide an unlimited supply of progenitor cells for the differentiation into mature spermatozoa.

Germ cell development involves epigenetic regulation of chromatin modifications and DNA methylation (Guan et al. 2012). The inheritance of the epigenetic modifications is reprogrammed in germ cells, but is relatively faithful in somatic cells. In mice, at embryonic day 7 when PGC specification occurs, level of genome wide DNA methylation, histone H3 lysine-9 di-methylation (H3K9me2) and lysine-27 tri-methylation (H3K27me3) are similar to those in surrounding somatic cells (Seki et al. 2007). Immediately after fate determination, the genome wide methylation patterns are erased and re-established during migration of PGCs to genital ridges (Schaefer et al. 2007). Once arriving at the genital ridge at embryonic day 11.5 in mice and by the fifth week of human development, PGCs undergo erasure and re-establishment of parental imprints during male and female gametogenesis before being passed to the next generation. Re-establishment occurs only after sex determination has been initiated, for review see (Saitou et al. 2012). This is a critical point, where the development of male and female germ cells goes differently. Under the influence of retinoic acid, produced by developing mesonephros, female germ cells enter meiosis. However, in developing testis Cyp26b1, a retinoic acid-degrading enzyme expressed by Sertoli cells prevents the meiosis in germ cells. As found in *cyp26b1* knockout mouse embryos, germ cells enter meiosis precociously (Bowles et al. 2006). Methylation of paternally imprinted genes is established in gonocytes up to the newborn. The newly established methylation imprints in gonocytes are then maintained through meiosis and passed to mature spermatozoa (Kato et al. 2007).

9.2.2 Classification of SSCs

In the mature testis, different germ cell types can be discriminated *in vivo*. Today scientists classify spermatogonia into different subtypes based on different morphologies. In the mature mouse testis, single type A spermatogonia are denoted as A_{single} (A_s), the most primitive cells located directly at the basal membrane of seminiferous tubules. Their percentage of all germ cells in the testes amounts to about 0.02–0.03% (Tegelenbosch and de Rooij 1993). Through symmetrical division either two daughter A_s cells or two A_{paired} (A_{pr}, two cell cysts) spermatogonia arise out of one mother A_s cell (Dym and Fawcett 1971; Greenbaum et al. 2006). A_{pr} spermatogonia are connected through a cytoplasmic bridge. They can divide furthermore and generate up to 32 jointly connected spermatogonia of the subtype $A_{aligned}$ (A_{al}, 4, 8, or 16 cell clusters) (de Rooij 2001). Further differentiation is orientated from the basal membrane of the seminiferous tubule towards the lumen. A_{al} spermatogonia differentiate into A_1, which subsequently go through six synchronous mitoses generating A_2, A_3, A_4, intermediate (IN) and B spermatogonia and spermatocytes which undergo meiosis and continue differentiation into spermatids and mature spermatozoa (de Rooij 2001). This linear model proposed in

1971 by Huckins (1971) and Oakberg (1971) suggests that stem cell capacity resides within A_s cells, whereas other A-type spermatogonia represent transit-amplifying progenitors, which divide uni-directionally to generate longer cysts (Fig. 9.1b). The simplicity of the linear model has led it to be widely accepted, despite claims that early cysts could reverse their paths and even replenish stem cells after tissue damage (Dym and Clermont 1970). However, the transplant assay demonstrate that A type spermatogonia, including the A_s, A_{pr}, and A_{al} spermatogonia have stem cell potential (Orwig et al. 2008). Recently, Nakagawa et al. evaluated this straight-forward linear model by applying the combination of lineage tracing and live imaging system and demonstrated that A_{pr} and A_{al} spermatogonia were not committed uni-directionally to differentiation but capable of reverting to A_s by fragmentation, and that the fate of individual spermatogonial populations was markedly altered during regeneration after damage (Fig. 9.1b) (Nakagawa et al. 2010).

In the adult human testis, there is still very little known about spermatogonial self-renewal. Clermont identified and characterized two spermatogonial subtypes of type A spermatogonia according to the staining pattern and the morphological characteristics of their nucleus nearly 50 years ago (Clermont 1963, 1966). They are referred to as dark type A spermatogonia (A_{dark}) and pale type A spermatogonia (A_{pale}). The A_{dark} spermatogonia have a discoid nucleus containing a deeply stained dust-like chromatin and a cavity with a pale stained material in the central part of the nucleus. Very often one or more nucleoli closely to the nuclear membrane are visible. The A_{pale} spermatogonia have an ovoid or discoid nucleus containing a pale staining granulated chromatin and showing one or two nulceoli attached to the nuclear envelope. In the human testis, beginning at approximately 2 months of age, gonocytes are replaced by A_{dark} and A_{pale} spermatogonia. According to Clermont's model both A_{dark} and A_{pale} spermatogonia are the stem cells. Specifically, A_{dark} spermatogonia are the presumptive reserve stem cells which represent a back-up of SSCs in the human testis and divide rarely but can be triggered to self-renew in case of injury or disease. In contrast, the A_{pale} spermatogonia represent the active stem cell pool which can self-renew and differentiate continuously to yield type B spermatogonia, which further differentiate into spermatocytes (Fig. 9.1d) (Clermont 1963, 1966, 1972). Therefore, in humans fewer mitotic steps are required to obtain spermatocytes and the efficiency of clonal expansion is very low in comparison to rodents (Bustos-Obregon et al. 1975; Johnson 1994; Johnson et al. 1999, 2001). Although this classification for human spermatogonia has been adopted by most researchers, the described model is challenged recently by Ehmcke and Schlatt, who suggested that A_{pale} spermatogonia underwent additional mitotic divisions (Ehmcke and Schlatt 2006). It has been demonstrated that in primates, a higher mitotic turnover is required from A_{pale} spermatogonia whose proliferation increases the total number of germ cells (Ehmcke et al. 2006). The role of stem cells is therefore limited to A_{dark} spermatogonia, which will replenish the progenitor compartment (A_{pale} spermatogonia) in case of cytotoxic or natural depletion (Ehmcke et al. 2006). Nevertheless, up to now, very little new information is available on the true identity of human SSCs and on the process of their self-renewal and differentiation (Dym et al. 2009).

9.3 Characteristics and Properties

9.3.1 Characteristics of Mouse SSCs

SSCs in rodents have been extensively characterized regarding the expression of marker genes and cell surface molecules. The surface phenotype of mouse undifferentiated spermatogonia, including SSCs is major histocompatibility complex (MHC) class 1 $(MHC\text{-}1)^{-}$ thymus cell antigen 1 $(Thy\text{-}1)^{low/+}$ c-Kit receptor tyrosine kinase $(c\text{-}Kit)^{-}$ β1-integrin $(ITGB1)^{+}$ α6-integrin $(ITGA6)^{+}$ αv-integrin $(ITGAV)^{-/dim}$ at all postnatal ages (Kubota et al. 2003, 2004a; Shinohara et al. 1999). SSCs are also positive for cell surface markers, such as Ep-CAM, CD9, glial cell-derived neurotrophic factor (GDNF) receptor (GFR)α1 and c-Ret receptor tyrosine kinase (RET), and an orphan adhesion-type G-protein-coupled receptor (GPR125) (Buageaw et al. 2005; Kubota et al. 2003; Naughton et al. 2006; Ryu et al. 2004; Seandel et al. 2007). Antibodies specific to the listed antigens allow enriching of SSCs by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS). Recently, a well-known cell adhesion molecule E-cadherin has been found to be a marker of mouse SSCs. E-cadherin appearance coincided with expression of another SSC marker promyelocytic leukemia zinc finger (PLZF), and its expression was decreased in c-Kit positive differentiating spermatogonia (Tokuda et al. 2007; Tolkunova et al. 2009). C-Kit is a hallmark for the more differentiated spermatogonia, including type $A_{1–4}$ spermatogonia (Yoshinaga et al. 1991).

SSCs have a common feature with other adult stem cells (for example, from the bone marrow or skeletal muscle), such as the ability to exclude DNA binding dye Hoechst. Therefore, SSCs can be identified by FACS after staining with Hoechst as so-called side population (Falciatori et al. 2004; Lassalle et al. 2004). RNA expression analysis demonstrated that the side population in testicular cells contains spermatogonial cells expressing germline stem cell markers α6-integrin and Stra8 (Lassalle et al. 2004). Hoechst efflux can be prevented by a specific ATP-binding cassette subfamily G member 2 (Abcg2 or Bcrp) inhibitor Ko143, suggesting that the 'side population' phenotype of SSCs is dependent on Abcg2 activity. The side population phenotype is also conferred by Abcg2 expression (Falciatori et al. 2004).

Recently, Nakagawa et al. propose that gene expression appears to be the better indicator of the fate of individual cells over the morphological criteria. PLZF and E-cadherin have essentially identical expression patterns and are found in all A_s, A_{pr} and A_{al} spermatogonia (Nakagawa et al. 2010) whereas GFRα1 mostly marks A_s or A_{pr} spermatogonia and neurogenin (NGN)3-positive cells are mainly A_{al} (Nakagawa et al. 2010; Yoshida et al. 2007a). A cytoplasmic protein encoded by the retinoic acid-responsive gene *Stra8*, is a specific marker for premeiotic spermatogonia and their progenitors (Giuili et al. 2002; Guan et al. 2006). Additionally, the pluripotency factor Lin28 marks also all the A_s, A_{pr} and A_{al} spermatogonia, and Lin28-positive cells exist as two subpopulations: NGN3-negative (high stem cell potential) and NGN3-positive (high differentiation commitment) cells (Zheng et al. 2009).

Another pluripotency factor Oct4 (POU5F1), a germline-specific transcriptional factor, is also expressed in mouse SSCs (Ko et al. 2009; Ohbo et al. 2003; Ohmura et al. 2004). Knockouts for several transcription factors, such as TAF4b (Falender et al. 2005), Ets variant gene 5 (Etv5) (Tyagi et al. 2009) and PLZF (Buaas et al. 2004; Costoya et al. 2004) resulted in impairment of spermatogonial compartment and therefore appear to be crucial for spermatogenesis. Mice with targeted disruption of *Etv5* showed total loss of undifferentiated spermatogonia resulting in a Sertoli cell-only phenotype and aspermia. Sertoli cells from Etv5 knockout mice revealed a significant decrease in expression of several chemokines. Chemotaxis assays demonstrated that migration of SSCs towards Sertoli cells from Etv5 knockout mice was significantly decreased in comparison to migration toward wild-type Sertoli cells. Rescue assays using recombinant chemokines indicated that C-C-motif ligand 9 (CCL9) facilitated Sertoli cell chemoattraction of SSCs, which express C-C-receptor type 1 (CCR1). This study also revealed that there was a protein-DNA interaction between Etv5 and CCL9, suggesting that Etv5 might be a direct regulator of CCL9 expression (Simon et al. 2010).

During last 10 years many labs performed transcriptome profiling studies in order to identify gene signatures characteristic for germ cells at different stages of development. These studies include time course of testis development during embryogenesis (Shima et al. 2004; Small et al. 2005), SSCs derived from neonatal testis (Hofmann et al. 2005) as well as studies of SSCs culture *in vitro* (Carlomagno et al. 2010; Hamra et al. 2004; Oatley et al. 2006). These studies describe new genes expressed in the SSCs as well as operating molecular pathways. For instance, Oatley et al. and Hoffmann et al. have discovered genes upregulated after stimulation of SSCs with GDNF (see below). Interestingly, genes with maximal change in expression are largely not coincident in these two studies. The reason could be the use of freshly isolated GFRα1 selected spermatogonia in one study (Hofmann et al. 2005) and long-term cultured SSCs in another (Oatley et al. 2006). Another reason could be different culture conditions, and conditions of GDNF stimulation (Caires et al. 2010). Therefore, new high-throughput screenings, especially employing combination of enriched SSCs and long-term cultured germ cells from the same origin would help to identify new SSC genes and signaling pathways involved in propagation and differentiation of these cells.

9.3.2 Characteristics of Human SSCs

Studies related to the fundamental questions of SSC biology have been mostly performed with mice and to a less extent with rats and pigs and very few with primates including humans. Interesting and challenging question is to what extent the discovered mechanisms are relevant for human SSCs. In the last 10 years, many studies showed that human and rodent spermatogonia shared many but not all phenotypic markers (Dym et al. 2009). Similar to mouse SSCs, human SSCs are positive for CD49f (α6-integrin), GPR125, CD9, CD90 (Thy-1), GFRα1, MAGE-4, and VASA,

and negative for CD117 (c-Kit) (Conrad et al. 2008; He et al. 2010, 2012; Izadyar et al. 2011; Sadri-Ardekani et al. 2009). PLZF, a crucial self-renewal factor of rodent SSCs was found in monkey A_{dark}/ A_{pale} (Hermann et al. 2007) and presumably human SSCs (Dym et al. 2009). It might be that function of PLZF is conserved between rodents and primates. For the enrichment of human SSCs, strategies developed for mouse SSCs including morphology-based selection, laminin selection and MACS using cell surface markers such as CD49f, GPR125, CD9 and SSEA4 have been applied in many studies (Conrad et al. 2008; Golestaneh et al. 2009b; He et al. 2010, 2012; Izadyar et al. 2011; Kossack et al. 2009; Lim et al. 2010; Mizrak et al. 2010; Sadri-Ardekani et al. 2009).

In contrast to mouse SSCs, human SSCs do not express CD29 (β1-integrin) (Izadyar et al. 2011). The mouse germline transcriptional factor Oct4 is not detected in adult human spermatogonia (Looijenga et al. 2003). In addition, other rodent markers, including NGN3, RET and Stra8 have not been studied in human spermatogonia. Further investigations to uncover the similarities and/or differences in spermatogonial phenotypes between humans and rodents are necessary. This will help us to understand molecular mechanisms controlling self-renewal and differentiation of SSCs.

9.3.3 SSC Niche

The term "stem cell niche" is used to describe the microenvironment in which stem cells are found. It, interacts with stem cells to regulate stem cell fate, and comprises cells, extracellular matrix components, and local soluble factors. The maintenance and differentiation of SSCs in adult mammalian testis take place in the seminiferous epithelium. Peritubular myoid cells and Sertoli cells are the structural basis for the SSC niche. Peritubular myoid cells form the outer layer of seminiferous tubules, whereas Sertoli cells encompass and nourish the germ cells forming the scaffolding structure of the seminiferous tubules. Peritubular myoid cells contribute to the contractile activity of testicular tubules and maintain mesenchymal-epithelial interactions with Sertoli cells both by cooperation in the deposition of extracellular matrix elements and by secretion of paracrine agonists, for example, PModS (Peritubular factor that Modulates Sertoli cell function) (Verhoeven et al. 2000). It has been reported that fibroblast growth factor (FGF) 2 and FGF9 can mediate mesenchymal-epithelial interactions of peritubular and Sertoli cells in the rat testis (El Ramy et al. 2005). However, peritubular myoid cells are present in all areas of the tubule basal membrane, and do not likely determine the location of the SSC niche (de Rooij 2009).

Sertoli cells play a key role in the formation of the niche for SSCs. Tight junctions formed between neighboring Sertoli cells constitute a protective blood-testis barrier, which separates seminiferous epithelium into basal and adluminal compartments. All the stages of spermatogonia before meiosis including SSCs reside in the basal compartment (de Rooij and Russell 2000; Fijak et al. 2011). Advanced meiotic

spermatocytes and all post-meiotic germ cells are located in the immune privileged adluminal compartment (de Rooij and Russell 2000; Fijak et al. 2011). In the absence of Sertoli cells, successful and complete spermatogenesis resulting in mature sperm has not been demonstrated in mammals. Accumulating data have shown that Sertoli cells provide the necessary ligands to spermatogonia, such as GDNF (Meng et al. 2000), SCF (Feng et al. 2000), FGF2 (Oatley and Brinster 2008), and the ligand of non-canonical Wnt pathway, Wnt5a (Yeh et al. 2011). These factors mediate external signals that define the proliferation rate and survival of spermatogonia and control spermatogenesis (see below).

The rate of spermatogenesis is controlled by sex hormones, but it appears that germ cells are affected by this regulation indirectly. Interestingly, transplantation experiments revealed that rat SSCs supported by mouse Sertoli cells were differentiating with the timing characteristic of the rat, and generated the spermatogenic structural pattern of the rat, demonstrating that the timing of the cell differentiation process of spermatogenesis was regulated by germ cells alone (França et al. 1998; Griswold 2007). However, somatic cells of the seminiferous epithelium define the efficiency of spermatogenesis and connect the process to the hormonal regulation. It is known that peritubular and Sertoli cells, but not germ cells are affected by androgens produced by Leydig cells, which reside in the interstitial tissue of the testis (Fijak et al. 2011). Previous studies showed enhanced colonization of SSCs in the testes of recipient mice treated with leuprolide which lowers testosterone levels (Ogawa et al. 1998).

Interestingly, small blood vessels running near the tubule wall or patches of Leydig cells determine the size of the niche (de Rooij 2009). Recently, Yoshida and colleagues performed imaging experiments analyzing the distribution of SSCs expressing GFP under the control of *NGN3* promoter in the basal compartment of seminiferous epithelium. They found that undifferentiated spermatogonia (A_s, A_{pr}, and A_{al}) clustered along blood vessels and interstitium, whereas the later A_1 and A_2 divisions occurred away of this original position. Within the zone facing the interstitium and blood vessels, stem cells exhibited a further preference for the branch points of the blood vessels (Yoshida et al. 2007b). It remains to be determined on what this SSC localization is dependent. It may be the components of the interstitial tissue that determine whether Sertoli cells produce factors to induce self-renewal and differentiation of SSCs. Furthermore, peritubular myoid cells and interstitial cell types and blood-born factors may also have direct effects on maintenance and/or differentiation of SSCs.

9.3.4 Signaling Pathways Involved in Self-Renewal of SSCs

The continual production of mature spermatozoa throughout the whole lifespan of the organism requires the maintenance of stem cells capable of self-renewal and differentiation. In the next part of the review we would like to focus on the most recent findings regarding signaling events controlling self-renewal of SSCs.

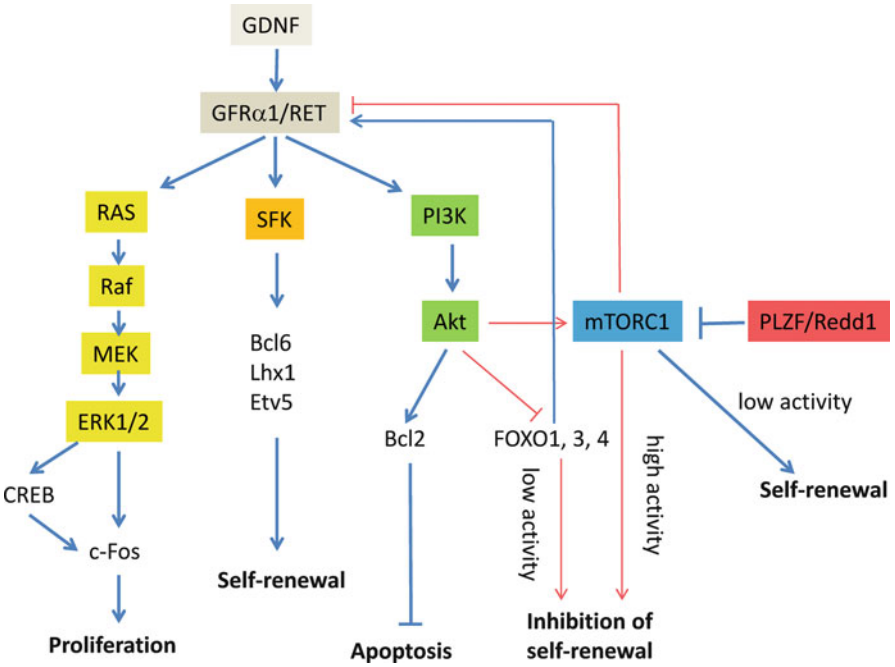


Fig. 9.2 GDNF mediated signaling pathways involved in self-renewal of SSCs. Briefly: GDNF is the most important factor for self-renewal of SSCs. It operates mainly through SFK and PI3K/Akt pathways controlling the expression of essential SSC genes (such as Etv5, Bcl6b, and Lhx1). In addition, GDNF can activate the canonical RAS/ERK1/2 pathway, which results in phosphorylation and activation of transcription factors such as CREB and c-Fos. Akt plays a controversial role in self-renewal of SSCs: on one hand it promotes self-renewal and inhibits apoptosis, on the other hand it phosphorylates and inactivates another important SSC factor, FOXO1. Additionally, PI3K-Akt might be also involved in activation of mTORC1 complex. PLZF counteracts excessive mTORC1 activity by controlling the expression of mTORC1 inhibitor Redd1. It is suggested that PI3K-Akt signaling must be carefully titrated in vivo to maintain SSC self-renewal and differentiation and that the Foxos and mTORC1 are pivotal intermediaries of this balance

9.3.4.1 Self-Renewal of SSCs Regulated by GDNF

GDNF is provided to SSCs by Sertoli cells and acts through a receptor heterodimer of RET and GFRα1 (Fig. 9.2) (Naughton et al. 2006). Intracellular events stimulated by GDNF in SSCs are the main focus of investigations in the area of SSCs, and multiple signaling pathways are induced by GDNF via its interaction with RET (for interests, please also see review (Caires et al. 2010)). Signaling initiated by GDNF is necessary for the maintenance of stem cells in the testis. Mutant mice with one null *GDNF* allele underwent SSC depletion, whereas transgenic males overexpressing GDNF accumulated undifferentiated spermatogonia in the testes (Meng et al. 2000). GDNF is shown to activate both Akt and Src family kinases (SFK). Cultivation of SSCs with pharmacological inhibitors of these kinases followed by transplantation analysis shows impairment of SSCs maintenance *in vitro* (Oatley et al. 2007).

It has been shown that, through the SFK signaling pathway, stimulation of SSCs with GDNF results in upregulation of *Bcl6b*, *Etv5* and *Lhx1* gene transcription whereas the expression of these genes is decreased in the absence of GDNF (Oatley et al. 2007). The expression of *Bcl6b*, *Etv5* and *Lhx1* is also identified in undifferentiated spermatogonia *in vivo*, and knockdown of these genes by small interfering RNAs reveals that all of them are essential for SSC maintenance *in vitro* (Oatley et al. 2007).

In another study, Akt kinase is rapidly phosphorylated in SSCs when GDNF is added to the medium, and the small-molecule inhibitor of Phosphoinositide 3-Kinase (PI3K) prevents SSC self-renewal. Furthermore, conditional activation of the myristoylated form of Akt in SSCs promotes their proliferation in the absence of GDNF (Lee et al. 2007). Later studies point to a strictly controlled activity of PI3K/Akt pathway in spermatogonia, which is in part explained by the function of downstream targets, such as FOXO family of transcription factors (Goertz et al. 2011; Salih and Brunet 2008). FOXO proteins are well known to regulate cellular growth and organismal longevity inducing a variety of cellular responses including cell-cycle arrest and cell death. FOXO1, FOXO3 and FOXO4 may be regulated by Akt-dependent phosphorylation, leading to their functional inactivation by the export from the nucleus (Salih and Brunet 2008). Recently, it was found that FOXO1 was expressed in gonocytes and spermatogonia. During first week of postnatal development FOXO1 changed the subcellular localization from cytoplasmic in gonocytes to nuclear in spermatogonia. Conditional ablation of FOXO1 in germ cells did not affect the formation of gonocytes, whereas the amount of spermatogonia and more differentiated male germ cells was diminished. The triple knockout of FOXOs 1, 3, and 4 resulted in even fewer germ cells suggesting a partial functional redundancy in the FOXO family. Microarray analysis revealed that expression of *RET* gene was diminished after Cre-mediated ablation of *FOXO1* in germ cells, thereby explaining, in part, the role of FOXO1 factor in spermatogonia (Goertz et al. 2011). Taken together, the data suggest that PI3K-Akt signaling must be carefully titrated *in vivo* to maintain SSC self-renewal and differentiation and argue that the Foxos are pivotal intermediaries of this balance (Fig. 9.2).

It was recently found that functional inactivation of PI3K catalytic subunit in mice was detrimental for expansion of differentiating spermatogonia. Knock-in mice bearing a catalytically inactive subunit of p110β K805R demonstrated a decreased amount of differentiated germ cells in the male. These knock-in mice did not reveal any impairment in the development of SSCs during postnatal development, and SSCs were also unaffected in adult animals. Moreover, GDNF induced Akt phosphorylation and proliferation in cultured SSCs was not affected by TGX221, a specific pharmacological inhibitor of p110β PI3K subunit, but decreased after the treatment with a less selective PI3K inhibitor PIK75 (mainly inhibiting p110α subunit). Thus, presumably p110α is the main PI3K isoform activated by GDNF. Most strikingly, spermatogonia derived from testis of knockout mice failed to respond to SCF stimulation. Therefore, it appears that PI3K subunit p110β is necessary for c-Kit-mediated induction of proliferation and differentiation of spermatogonia (Ciraolo et al. 2010).

In addition, GDNF can activate the canonical RAS/ERK1/2 pathway, which results in phosphorylation and activation of transcription factors such as CREB1, ATF1, CREM and c-FOS (He et al. 2008). He and colleagues also demonstrated that *ERK 1/2* (*MAPK 1/3*) was up-regulated in isolated human SSCs when cultured for 2 weeks in media containing GDNF, and phosphorylated ERK1/2 was increased in cultured cells compared to freshly isolated cells (Fig. 9.2) (He et al. 2010). It appears that GDNF role is conserved between rodents and humans since GDNF allowed to obtain a short-term culture of presumable human SSCs (He et al. 2012).

9.3.4.2 Self-Renewal of SSCs Regulated by the mTORC1-PLZF Interaction

As discussed above, the PLZF protein expression was observed in both human and mouse undifferentiated spermatogonia (Hermann et al. 2007; Nakagawa et al. 2010), morphological types believed to be *in vivo* counterparts of SSCs *in vitro* (Buaas et al. 2004). A nonsense mutation in PLZF encoded by *Zfp145* gene has been determined in luxoid mutant mouse strain, which was previously found to be male infertile. Luxoid mice demonstrate a progressive loss of germ cells in the seminiferous tubules with the age. Phenotype similar to luxoid has been found in mice after targeting in the *Zfp145* gene locus, the gonocyte numbers were not decreased but germ cells were eliminating with age and amount of sperm was decreased dramatically (Costoya et al. 2004). These papers suggest that although dispensable for germ cell development in embryogenesis and postnatal period, PLZF becomes important for the maintenance of SSC population in testis. A recent work uncovered one of the functions of PLZF in SSCs. It turns out that SSCs lacking PLZF have enhanced activity of molecular target of rapamycin complex 1 (mTORC1), a key mediator of cell growth. PLZF opposes mTORC1 activity by inducing expression of the mTORC1 inhibitor Redd1 (Fig. 9.2). Increased mTORC1 activation in $PLZF^{-/-}$ SSCs inhibits their response to GDNF via negative feedback at the level of the GDNF receptors, GFRα1 and c-RET. The data also show that $PLZF^{-/-}$ SSCs have increased cell size compared to control, however this change can be prevented by rapamycin, a small molecule inhibitor of mTORC1 complex. Furthermore, inhibition of mTORC1 via rapamycin attenuates $PLZF^{-/-}$ SSC defects and enhances wild-type SSC activity. The authors suggest that the mTORC1-PLZF functional interaction is a critical rheostat for the maintenance of the spermatogonial pool and negative feedback from mTORC1 to the GDNF receptor balances SSC growth with self-renewal (Fig. 9.2) (Hobbs et al. 2010).

9.3.4.3 SSC Self-Renewal Controlled by Wnt Signaling

It is known that Wnt signaling promotes self-renewal of various stem cell types (Reya and Clevers 2005). Wnts and their receptors Frizzleds (Fzds) are expressed in mouse spermatogonia (Golestaneh et al. 2009a). High expression of *Fzd3* was found in GFRα1-positive SSCs while only a very low expression of *Fzd* was found in the

c-Kit-positive differentiating spermatogonial cells. Wnt3a and Wnt10b, both activators of canonical Wnt signaling, increased cell proliferation in primary mouse SSC culture (Golestaneh et al. 2009a). However, study of Yeh and co-authors suggest that canonical Wnt signaling would rather promote differentiating spermatogonia than true SSCs. They showed that non-canonical Wnt5a supported self-renewal of mouse SSCs in a β-catenin-independent manner (Yeh et al. 2011). Wnt5a expression was restricted to Sertoli cells in mouse testes and potential Wnt5a receptors Fzd5, Fzd7 and ROR2 were detected at the cell surface of SSCs. It was shown that the inhibition of β-catenin signaling via application of Dickkopf-1 (Dkk1), which specifically blocks β-catenin signaling by binding to LRP5/6, did not affect SSC activity. However, secreted frizzled-related protein 1 (sFRP1), which inhibits both β-catenin-dependent and -independent signaling by binding Wnt ligands led to a dose-dependent reduction of SSC activity. They found out that Wnt5a promoted SSC maintenance by supporting cell survival, and this pro-survival effect of Wnt5a was abolished by the inhibition of c-Jun N-terminal kinase (JNK) signaling. In addition, Wnt5a significantly increased JNK-*P* levels. Moreover, it was found that cells with activated β-catenin signaling had lost SSC function suggesting that canonical Wnt pathway might be activated during early differentiation of SSCs. Therefore Wnt5a can be considered as a new member besides other SSC niche factors, such as GDNF and FGF2 (Yeh et al. 2011).

9.3.4.4 Integrins

Integrin-mediated cell adhesion to extracellular matrix plays an important role in regulating stem cell function and maintenance. In particular, integrins help to define and shape the stem cell niche (Ellis and Tanentzapf 2010). SSCs reside at the basal membrane of seminiferous tubules, and are capable to bind to laminin. This feature is used for isolation of SSCs from testes of mice (Guan et al. 2009) and rats (Hamra et al. 2008) suggesting that SSCs have a set of receptors for extracellular matrix. As discussed above, expression of certain integrin subunits is characteristic of SSCs. α6-integrin has been identified as cell surface marker of both rodent (Kubota et al. 2003; Ryu et al. 2004) and human SSCs (He et al. 2012). β1-integrin was discovered to be important for a proper homing of SSCs to the basal membrane of the seminiferous tubules after SSC transplantation (de Rooij et al. 2008; Kanatsu-Shinohara et al. 2008). Ablation of β1-integrin in SSCs influences their ability to colonize the recipient testis *in vivo* and to bind to laminin *in vitro* indicating that β1-integrin plays an important role in SSC function.

9.3.5 SSC Culture Conditions

Development of conditions for long-term cultivation of SSCs *in vitro* is important to study the fundamental questions concerning spermatogonial lineage and to make

the use of these cells a valid clinical option. The establishment of SSC culutre requires the layer of feeder cells and appropriate medium containing all necessary supplements and growth factors. Initially, there were studies using Sertoli cell lines as feeder cells (Hamra et al. 2004). However, the best self-renewal of mouse SSCs was achieved with mouse embryonic fibroblasts (MEFs) primarily derived from day 13.5 embryos (Guan et al. 2009). The concentration of feeder cells on the plate is an important point for successful establishment of SSC cultures. The optimal concentration of feeder cells is estimated with $20–50 \times 10^3$ cells per cm^2. Whereas mouse SSCs can be cultured in the presence of fetal calf serum, the establishment of long-term culture of rat SSCs requires serum-free chemically defined culture medium (Wu et al. 2009b). Presumably this protocol with slight modifications might be used for cultivation of SSCs from other species. Previous studies show that SSCs cultivated in this serum-free medium allows propagation of rat SSCs *in vitro* and ensures the maintenance of potential of cultured SSCs to restore spermatogenesis after transplantation (Wu et al. 2009b). SSCs after *in vitro* propagation may be frozen in a usual freezing solution, and the freezing and thawing procedure is simple and does not differ from other routine cell lines. After thawing, the SSCs maintain their characteristics. This allows the use of cultured SSCs for the infertile treatment of patients after cancer therapy.

Although intense interest and subsequent research surrounds the regenerative potential of human SSCs, only until recently two studies report the *in vitro* long-term propagation of human SSCs isolated from patients undergoing orchiectomy for treatment of prostate cancer for up to 15 weeks (Sadri-Ardekani et al. 2009), and from testicular tissues of patients with obstructive or non-obstructive azoospermia for more than 6 months (Lim et al. 2010). However, both studies demonstrate that human SSCs proliferate rather slowly, which may limit their clinical applications. Of note, human and rodent spermatogonia share many but not all phenotypic markers, and cultivation of SSCs from both species needs application of GDNF and FGF2. This indicates that some common molecular mechanisms controlling self-renewal of SSCs may exist in both rodents and humans. Future research on optimization of culture conditions for human SSCs needs to test new factors known for regulating self-renewal of mouse SSCs. For example, inhibition of mTORC1 might be useful in order to get a stable human SSC culture. Wnt signaling molecules need to be tested in human SSC culture. However, one needs to keep in mind that characteristics of human SSCs also differ from rodent SSCs *in vivo* in some extent suggesting that the establishment of human SSC culture might also need additional factors, which are not essential for rodent SSCs.

9.3.6 Pluripotency and SSCs

It is well accepted that SSCs are unipotent when they are located within the testis. However, in the last couple of years many studies demonstrate that mouse SSCs cultured *in vitro* acquire a remarkable potential plasticity once they are removed

from their *in vivo* niche and can be reprogrammed by culture conditions alone into pluripotent stem cells (Guan et al. 2006; Kanatsu-Shinohara et al. 2004; Ko et al. 2009; Seandel et al. 2007) suggesting that pluripotency is kept in the adult male germline.

The first report on generation of germline cell-derived pluripotent stem cells can be traced back to 1992. When PGCs are cultured in the presence of SCF, leukemia inhibitory factors (LIF) and FGF2, they can become pluripotent stem cells, so called embryonic germ cells (EGCs) (Matsui et al. 1992; Resnick et al. 1992). They show similar characteristics as embryonic stem cells (ESCs), which are derived from inner cell mass of the early blastocyst, can spontaneously differentiate into multiple cell phenotypes *in vitro*, and form teratomas in nude mice (Matsui et al. 1992). In 1998, human EGCs were obtained from human PGCs of 5- to 11-week embryos exposed to the same growth factors (Shamblott et al. 1998). In 2004, Shinohara and colleagues showed that SSCs isolated from mouse neonatal testis could be reprogrammed to pluripotent GSCs by culture conditions. These pluripotent germline stem cells show a morphological phenotype similar to ESCs, and can be maintained *in vitro* for a long time period and differentiated into various cell types both *in vivo* and *in vitro*. However, Shinohara and colleagues could not derive ES-like cells from SSCs of adult mice (Kanatsu-Shinohara et al. 2004). Two years later, we for the first time demonstrated that mouse adult spermatogonia were able to be reprogrammed into ES-like pluripotent GSCs (Guan et al. 2006). Similar to mouse ESCs, the generated pluripotent GSCs express the cell surface marker SSEA-1, and transcription factors Oct4, Sox2, Nanog and Rex-1 and are also positive for alkaline phosphatase (Guan et al. 2006). It is worthy to mention that reprogramming of adult spermatogonia into pluripotent GSCs takes place spontaneously in culture. There is neither the addition of oncogenes nor the use of virus systems as described for generation of induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). Our study was then confirmed by several groups showing that adult mouse spermatogonia and/or their progenitors could indeed form pluripotent ES-like cells *in vitro* (Izadyar et al. 2008; Ko et al. 2009; Seandel et al. 2007). Seandel et al. generated pluripotent GSCs from GPR125 positive spermatogonia (Seandel et al. 2007) whereas two other studies established pluripotent GSC cultures from Oct-EGFP positive SSCs (Izadyar et al. 2008; Ko et al. 2009). Although isolation methods of SSCs differ among these studies, the generated pluripotent GSCs exhibit similar characteristics as ESCs.

Following these mouse studies, many efforts have been put in the human SSC research. Recently, several studies reported the generation of pluripotent GSCs from human testis, and showed that the cultured human GSCs can differentiate into derivatives of all three germ layers *in vitro* (Conrad et al. 2008; Golestaneh et al. 2009b; Kossack et al. 2009; Mizrak et al. 2010). However, in comparison to human ESCs, these cells do not fulfill all criteria for pluripotency: (1) they show a limited potential for teratoma formation; (2) expression levels of pluripotency-specific genes such as *OCT4*, *NANOG* and *SOX2* in generated pluripotent GSCs are much lower than that in human ESCs; and (3) the promoters of *OCT4* and *NANOG* genes are only partially demethylated compared to human ESCs. The possible explanation

could be that the cells have not been completely reprogrammed to the pluripotent state under the conditions used in those studies. Moreover, the cellular origin of these GSC cultures has been questioned. In all of these studies, the isolated cells were not analyzed for the expression of testicular somatic cell-specific genes to rule out the presence of other cell types, and the markers used for characterization of the isolated cells are not specific for human SSCs (Dym et al. 2009). It was demonstrated that the global gene expression profile in one of the reported human pluripotent GSC cultures was similar to that of human testicular fibroblast cells but not to human SSCs (Ko et al. 2010). Notably, GPR125 was also used for isolation of human SSCs (He et al. 2010). However, conversion of human GPR125-postive cells into pluripotent GSCs has not been reported, thus, further investigations are necessary.

9.4 Differentiation Capacities and Their Progenitors

9.4.1 Differentiation Capacities of SSCs In Vivo and In Vitro

Differentiation potential of *in vitro* cultured SSCs can be proved by their potential to restore the spermatogenesis after transplantation of these cells into the seminiferous tubules of infertile recipient mice. Previous studies showed that mouse SSCs were able to repopulate the seminiferous tubules 2 months after they were injected into the germ cell-depleted testis (Brinster and Zimmermann 1994). In this study, male mice were treated with the alkylating antineoplastic agent busulfan, which destroys SSCs and thus leads to a disruption of spermatogenesis. Testes of busulfan treated mice usually do not contain mature spermatozoa or even spermatogonia. After injection of SSCs from transgene *lacZ* (*β-Galactosidase gene*) donor mice into busulfan treated recipient mice functional spermatogenesis could be observed. Because the donor cells carried a transgene that produced β-Galactosidase in spermatids, these cells were identified by a blue staining after X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) treatment. The organization of the spermatogenic stages within the seminiferous tubules was normal and mature spermatozoa were produced. These data suggest that the transferred SSCs are able to colonize the seminiferous tubules of infertile mice and subsequently undergo self-renewal division to support spermatogenesis. The repopulation capacity was also proved for SSCs derived from the rat (Hamra et al. 2002). Human SSCs were found as singlets or doublets on the seminiferous tubule basement membrane 3–6 months after transplantation of human SSCs to testes of immunodeficient mice. These results indicate that human spermatogonia home to the basement membrane of the mouse recipient seminiferous tubule and are maintained as germ cells, but are unable to differentiate (Nagano et al. 2002; Wu et al. 2009a). Interestingly, when human spermatogonia were injected together with human testicular somatic cells, the greater number of singlets and doublets, the larger groups of human germ cells, and particularly the presence of dividing germ cells could be observed in mouse seminiferous tubules.

These data indicate that human testicular somatic cells enhance the ability of human spermatogonia to colonize the mouse seminiferous tubule (Wu et al. 2009a). The ability of human SSCs to restore spermatogenesis would be the foundation for the treatment of men infertility.

In spite of the ability to differentiate *in vivo* in the testis, the complete process of spermatogenesis has not been achieved from SSCs under *in vitro* cell culture conditions. *In vitro* differentiation of SSCs and the use of *in vitro* derived male haploid gametes for intracytoplasmic sperm injection could be an option to restore fertility. The most advanced differentiation has been achieved using mouse SSCs. Treatment of mouse SSCs with SCF induced the formation of spermatocytes after 1 week. The latest differentiation stage observed was round spermatids, which appeared in culture after 3 weeks of SCF treatment (Feng et al. 2002). However, the study has not been repeated by another group or used to study signaling during differentiation of germ cells. One reason might be that cells used in this study were immortalized by overexpression of TERT (telomerase reverse transcriptase) and could be therefore different from primary SSCs (Feng et al. 2002).

It is worth to mention that there is only a minor percent of SSCs bearing self-renewal and capable of testis colonization under *in vitro* optimal culture conditions. Under the established culture conditions, SSCs consistently give rise to both new SSCs and differentiating progeny. The differentiating progeny have lost stemness and unlimited self-renewal capacity, but have developed functional intercellular bridges. Twin daughter cells of single SSCs often undergo self-renewal and differentiation side by side even though they have been exposed to virtually identical microenvironments. Moreover, quantitative experimental measurements and mathematical modeling indicates that fate decision is stochastic, with constant probability (Wu et al. 2009c). Therefore, SSCs seem to have an in-built program of self-renewal and differentiation, and probably the extent of survival of differentiating cells might be affected by external factors.

A recent paper showed that BMP4 induced the expression of early differentiation factor c-Kit in a rat SSC-like cell line (Carlomagno et al. 2010). In addition, vitamin A derivatives, such as retinoic acid are absolutely essential for the initiation of meiosis in germ cells in the testis. Mice and rats, subjected to vitamin A deficient diet for several weeks become infertile and contain no mature germ cells in testis. The injection of retinol induced the synchronous onset of spermatogenesis (Van Pelt and De Rooij 1990a, b). *Stra8* gene is one of the targets of retinoic acid signaling, and is essential for meiosis in male and female germ cells (Anderson et al. 2008; Mark et al. 2008). Expression of Stra8 and c-Kit was induced in spermatogonia both *in vivo* and *in vitro* (Zhou et al. 2008a, b). Therefore, retinoic acid might be an essential component for *in vitro* differentiation of SSCs. Future works should test whether treatment of SSCs with retinoic acid and other factors mentioned above can induce the initiation of meiosis *in vitro*, and should identify the specific factors required for a more efficient and complete *in vitro* spermatogenesis. Moreover, culture medium supporting the survival of spermatocytes and spermatids needs to be developed.

9.4.2 *Differentiation of Pluripotent GSCs In Vitro*

As we know, pluripotent GSCs, similar to ESCs are able to spontaneously differentiate into derivatives of all three embryonic germ layers when they are removed from feeder cells (Cheng et al. 2012; Fagoonee et al. 2010; Guan et al. 2006, 2007; Streckfuss-Bomeke et al. 2009). We show that cardiomyocytes derived from pluripotent GSCs express cardiac-specific L-type Ca^{2+} channels and respond to Ca^{2+} channel-modulating drugs. Four different types of action potentials characteristic of pacemaker-, ventricle-, atrial- and Purkinje-like cardiomyocytes are observed. The cardiomyocytes derived from pluripotent GSCs also exhibit functional gap junctions as well as an intact calcium cycling (Guan et al. 2007). We also demonstrate that pluripotent GSCs can be differentiated into $Flk1^+$ cells with an efficiency of 35% when co-cultured with OP9 stromal cells (Cheng et al. 2012). $Flk1^+$ cells are multipotent cardiovascular progenitors which can contribute to the cardiomyocyte, endothelial, and vascular smooth muscle lineages (Kattman et al. 2006; Yang et al. 2008). $Flk1^+$ cells derived from pluripotent GSCs express cardiovascular progenitor markers Isl-1, Nkx2.5 and brachyury, and are able to further differentiate into functional cardiomyocytes as well as functional endothelial cells. In addition, the differentiated contractile cells express sodium, potassium and calcium channels (Baba et al. 2007). These data indicate that GSC-derived cardiovascular progenitors as well as functional cardiomyocytes and endothelial cells may provide a useful source of cardiovascular cells for studying basic mechanisms of cardiogenesis and vasculogenesis and for cardiovascular regeneration.

Besides functional cardiomyocytes, mouse pluripotent GSCs can differentiate into neural progenitors under specific culture conditions, which can further differentiate into functional neurons (GABAergic, glutamatergic, serotonergic, and dopaminergic neurons) and glial cells (astrocytes and oligodendrocytes). Electrophysiological recordings of passive and active membrane properties and postsynaptic currents demonstrate the maturation of neural precursor cells into functional neurons and glial cells (Streckfuss-Bomeke et al. 2009). Therefore, pluripotent GSC-derived neural precursors and functional neurons and glial cells constitute a promising cell source for the treatment of many different nervous system disorders.

Several groups have reported the expression of the early hepatic marker, α-fetoprotein, in embryoid bodies generated from mouse pluripotent GSCs (Fagoonee et al. 2010; Guan et al. 2006). Furthermore, metabolically active hepatocytes can be derived from pluripotent GSCs *in vitro*, which are capable of albumin and haptoglobin secretion, urea synthesis, glycogen storage, and indocyanine green uptake (Fagoonee et al. 2010). The pluripotent GSC-derived hepatocytes were found to be closer to fetal hepatocytes than adult hepatocytes (Fagoonee et al. 2010). The functional hepatocytes may be a useful cell source for liver regeneration.

9.5 Potential Applications for Therapies

9.5.1 Therapeutic Application of SSCs in Male Infertility

Male infertility can be caused by genetic defects of the endocrine system, by defects in the development of the urogenital system or by defects in gametogenesis, cryptorchidism or erectile dysfunction. In addition, there are also secondary or acquired causes of infertility due to tubal disease or exposure to gonadotoxins from the environment (Matzuk and Lamb 2008). Among these causes, testicular cancer as well as the effect of radiotherapy or chemotherapy used in cancer treatment can also result in male infertility. In testes, SSCs and differentiating spermatogonia divide most actively and are therefore extremely sensitive towards cytotoxic agents (Meistrich 1993). In contrast, Leydig and Sertoli cells can survive most cytotoxic therapies and may sustain a functional damage because of lower proliferation rate in adults. After cytotoxic therapies, seminiferous tubules contain only Sertoli cells, whereas germ cells appear to be absent (Shetty and Meistrich 2005). This could be the result of the deletion of SSCs and/or the loss of the ability of the remaining Sertoli cells to support the self-renewal and differentiation of a few surviving SSCs. At lower doses of cytotoxic agents recovery of spermatogenesis can be observed several months after termination of the treatment. At higher doses, however, azoospermia can be prolonged or even permanent. Adult male cancer patients have the ability of cryopreservation of their semen prior to chemo- or radiotherapy. This allows them to have children by artificial insemination after successful cancer treatment. However, prepubertal boys treated with high-dose chemotherapy, total body irradiation and/or irradiation involving the genital region cannot benefit from this approach since spermatogenesis at that age is not yet completed. At present, the only option for fertility preservation could be SSC preservation. Therefore, it is necessary to take a biopsy of testis tissue before chemo- or radiotherapy (Fig. 9.3). The establishment of isolation and long-term cultivation of human SSCs *in vitro*, as well as cryopreservation of these cells provides the opportunity for clinical applications with regard to the treatment of male infertility. After successful cancer treatment the cultivated SSCs could be transplanted back into the seminiferous tubules of the patient (Geens et al. 2008). Autologous intra-testicular transplantation of SSCs is a hypothetical option that is currently thoroughly studied by a few research groups, mainly in rodent models. Already in 1994, Brinster and Zimmermann (1994) could show that successful restoration of spermatogenesis occurs in infertile mice after injection of SSCs into seminiferous tubules. The transplantation of SSCs has been shown to produce live offspring in mice (Goossens et al. 2003, 2006). The success of these transplantation experiments in rodents suggests therapeutic potential for the patients. However, some safety concerns related to this technique should be taken into consideration, the risk of transmitting tumor cells back to the patient and other aspects of the procedure, like germ cell retrieval, cell sorting and preservation, still need to be optimized prior to clinical applications in men.

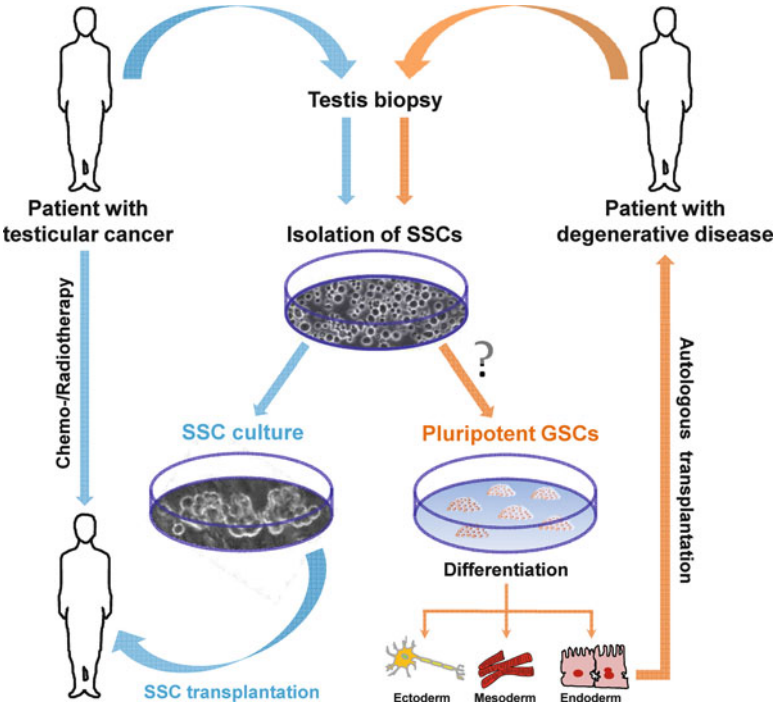


Fig. 9.3 Potential application of SSCs in treatment of infertility after chemo-/radiotherapy in patients with testicular cancer and potential application of SSC-derived pluripotent GSCs in regenerative medicinen. *SSCs* spermatogonial stem cells, *GSCs* germline stem cells

9.5.2 Therapeutic Applications of Pluripotent GSCs

As mentioned above (see Sects. 9.3.6 and 9.4.2), mouse SSCs can be converted into pluripotent GSCs *in vitro* under defined culture conditions without addition of genes. Two characteristics of pluripotent stem cells make them interesting for regenerative medicine: their high proliferation rate and their ability to differentiate into all different cell types of the body. The establishment of protocols for a directed differentiation of pluripotent stem cells allows the production of any somatic cell type which is needed. The main focus of stem cell research has been on cell therapy for pathological conditions with no current methods of treatment, such as neurodegenerative diseases, heart attacks, retinal dysfunction and lung and liver disease. The idea is to replace diseased or damaged tissue by using somatic cells derived from pluripotent stem cells. The overall aim is to develop methods of application either of pure cell populations or of whole tissue parts to the diseased organ. Transplantation of $Flk1^+$ cardiovascular progenitor cells derived from mouse pluripotent GSCs directly into the ischemic heart of mouse resulted in the improvement of cardiac function by promoting angiogenesis as well as postponing host cell death (Iwasa et al. 2010). Although mouse pluripotent GSCs can differentiate into functional neurons, glial cells as well as hepatocytes, the *in vivo* colonization

capacity of hepatocytes in mouse models of liver diseases, or regeneration potential of functional neurons in the damaged brain remains to be demonstrated. These mouse *in vitro* and *in vivo* studies pave the way for therapeutic application of pluripotent GSCs; however, up to now there are no evidences showing that so far generated human pluripotent GSCs are able to differentiate into functional cardiomyocytes, neurons, or hepatocytes. The drawback of so far generated human pluripotent GSCs is that they may be not truly pluripotent and thus their differentiation potential is limited. Therefore, development of a culture system for establishing truly pluripotent human GSCs is of paramount importance. Optimizing culture conditions by application of small molecules may facilitate the generation of pluripotent GSCs (Zhu et al. 2011).

With regard to clinical applications, the use of patient-specific stem cells, such as human pluripotent GSCs, for autologous stem cell-based therapies avoids immunological and ethical problems related to human ESCs. SSCs could be obtained from testis biopsies, cultivated *in vitro* and converted into pluripotent stem cells (Fig. 9.3). These cells can then be differentiated into somatic cells and transplanted back into the patient. Thus, the risk of immunological rejection is reduced onto a minimum and the patient is not dependent on immune suppressant drugs. Furthermore, patient-specific pluripotent GSCs could be used for somatic gene therapy. Cells from a patient with a genetic defect could be repaired *in vitro* and transplanted back into the patient. In a mouse model, it has been shown that one of the mutated alleles could be repaired by homologous recombination in ESCs coming from the immune-deficient mice. Hematopoietic precursor cells were derived by *in vitro* differentiation from the repaired ESCs and transplanted into the mutant mice. Mature myeloid and lymphoid cells as well as immunoglobulins became detectable several weeks after transplantation. The immune system of immunodeficient mice was re-established (Rideout et al. 2002).

Pluripotent GSCs similar to ESCs are pluripotent and are able to differentiate into derivatives of all three germ layers. So far, many studies using human pluripotent ESCs in organ regeneration have been performed and discussed, for interests and more details, please see reviews (Kung and Forbes 2009; Ronaghi et al. 2010; Shiba et al. 2009; Varanou et al. 2008). The first FDA approval (http://www.fda.gov) for the preclinical usage of differentiated human ESCs for the treatment of spinal cord injury makes human ESCs a very attractive source for clinical applications. However, in August 2009, the FDA put a clinical hold on human ESC clinical trials because further characterization of differentiated cells and more nonclinical trials/applications of human ESC-derived neural cells into animal models have been requested (Ronaghi et al. 2010).

9.6 Conclusions and Future Development in Research

SSCs constitute one of the most important stem cell systems in the adult body. SSCs are unipotent and respond for spermatogenesis in the male as they can only differentiate into sperms within the testicular niche. Methods for isolation and cultivation of SSCs from the rodent testis have been well established. Rodent SSCs can be expanded *in vitro* for a long term without loss of their properties. Only recently some studies

report that human SSCs can be isolated from adult testis biopsies and propagated *in vitro*. This provides the opportunity to develop therapeutic strategies and re-initiate spermatogenesis for the patients who become infertile after cancer therapy.

Since pluripotent stem cells can be generated from PGCs, neonatal and adult SSCs, we believe that the pluripotency is maintained in the male germline. The SSCs can be spontaneously reprogrammed into pluripotent GSCs when they are removed from their *in vivo* niche and cultured *in vitro* under specific conditions. The advantage of pluripotent GSCs over iPSCs is that conversion of SSCs into pluripotent GSCs does not require addition of genes using virus system, which may avoid unpredictable genetic dysfunction. This makes them a safer cell source for autologous transplantation than iPSCs. In addition, this may also circumvent the ethical and immunological problems associated with human ESCs. Future works should focus on optimizing protocols for isolation and long-term culture of human SSCs as well as establishing culture conditions for generation of pluripotent GSCs fulfilling all criteria for pluripotency. In addition, it is of paramount importance to identify the cell type in the testis that is capable of the conversion into the pluripotent stem cells, presumably it is the human SSCs, but this needs to be determined. Xenotransplantation studies might be used to confirm the function of human SSCs (Hermann et al. 2010). This will bring the realization of personalized regenerative medicines closer. However, this approach is suitable only for men, the half of the world's population. For women, derivation of parthenogenetic stem cells from the woman whose unfertilized eggs are artificially activated may provide another potential source for cell-based autologous transplantation therapy (Turovets et al. 2011).

Finally, successful development of pluripotent stem cell-based replacement strategies for various diseases needs to address three important questions: (1) how to generate an adequate number of cells sufficient for active improvement of organ function? More intensive work to obtain a better understanding of stem cell differentiation pathways and to improve differentiation protocols of pluripotent stem cells may help to find out a solution for it. (2) How to improve the survival of transplanted cells in the damaged organ upon transplantation? (3) How to eliminate residual undifferentiated cells from differentiated cells which are destined for *in vivo* transplantation, as these undifferentiated cells may form tumors? Moreover, the ideal source of stem cells for efficient and safe cell replacement has remained a challenging issue that requires more investigation.

Acknowledgments The authors studies were funded by Bundesministerium für Bildung und Forschung (01GN0822 and 01GN0957) and Deutsche Forschungsgemeinschaft (GU 595/2-1). The authors would like to thank Dr. Felix Bremmer (Dept. Pathology, University of Göttingen) for providing us the histochemical sections of human testicular tissue.

References

Anderson R, Fässler R, Georges-Labouesse E et al (1999) Mouse primordial germ cells lacking beta1 integrins enter the germline but fail to migrate normally to the gonads. Development 126(8):1655–1664

Anderson EL, Baltus AE, Roepers-Gajadien HL et al (2008) Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. Proc Natl Acad Sci USA 105(39):14976–14980
Baba S, Heike T, Umeda K et al (2007) Generation of cardiac and endothelial cells from neonatal mouse testis-derived multipotent germline stem cells. Stem Cells 25(6):1375–1383
Bowles J, Knight D, Smith C et al (2006) Retinoid signaling determines germ cell fate in mice. Science 312(5773):596–600
Brinster RL (2007) Male germline stem cells: from mice to men. Science 316(5823):404–405
Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci USA 91(24):11298–11302
Buaas FW, Kirsh AL, Sharma M et al (2004) Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 36(6):647–652
Buageaw A, Sukhwani M, Ben-Yehudah A et al (2005) GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. Biol Reprod 73(5):1011–1016
Buehr M, McLaren A, Bartley A et al (1993) Proliferation and migration of primordial germ cells in We/We mouse embryos. Dev Dyn 198(3):182–189
Bustos-Obregon E, Courot M, Flechon JE et al (1975) Morphological appraisal of gametogenesis. Spermatogenetic process in mammals with particular reference to man. Andrologia 7(2):141–163
Caires K, Broady J, McLean D (2010) Maintaining the male germline: regulation of spermatogonial stem cells. J Endocrinol 205(2):133–145
Carlomagno G, van Bragt MPA, Korver CM et al (2010) BMP4-induced differentiation of a rat spermatogonial stem cell line causes changes in its cell adhesion properties. Biol Reprod 83(5):742–749
Cheng I-F, Kaiser D, Huebscher D et al (2012) Differentiation of multipotent adult germline stem cells derived from mouse testis into functional endothelial cells. J Vasc Res 49(3):207–220
Ciraolo E, Morello F, Hobbs RM et al (2010) Essential role of the p110beta subunit of phosphoinositide 3-OH kinase in male fertility. Mol Biol Cell 21(5):704–711
Clermont Y (1963) The cycle of the seminiferous epithelium in man. Am J Anat 112:35–51
Clermont Y (1966) Renewal of spermatogonia in man. Am J Anat 118(2):509–524
Clermont Y (1972) Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 52(1):198–236
Conrad S, Renninger M, Hennenlotter J et al (2008) Generation of pluripotent stem cells from adult human testis. Nature 456(7220):344–349
Costoya JA, Hobbs RM, Barna M et al (2004) Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 36(6):653–659
De Felici M, Dolci S (1989) In vitro adhesion of mouse fetal germ cells to extracellular matrix components. Cell Differ Dev 26(2):87–96
De Felici M, Scaldaferri ML, Lobascio M et al (2004) Experimental approaches to the study of primordial germ cell lineage and proliferation. Hum Reprod Update 10(3):197–206
de Rooij DG (1998) Stem cells in the testis. Int J Exp Pathol 79(2):67–80
de Rooij DG (2001) Proliferation and differentiation of spermatogonial stem cells. Reproduction 121(3):347–354
de Rooij DG (2009) The spermatogonial stem cell niche. Microsc Res Tech 72(8):580–585
de Rooij DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21(6):776–798
de Rooij DG, Repping S, van Pelt AM (2008) Role for adhesion molecules in the spermatogonial stem cell niche. Cell Stem Cell 3(5):467–468
Donovan P, de Miguel M (2007) Gonocyte development and differentiation. In: Skinner MK, Griswold MD (eds) Sertoli cell biology. Elsevier, Amsterdam, pp 417–435
Dym M, Clermont Y (1970) Role of spermatogonia in the repair of the seminiferous epithelium following x-irradiation of the rat testis. Am J Anat 128(3):265–282
Dym M, Fawcett DW (1971) Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. Biol Reprod 4(2):195–215

Dym M, Kokkinaki M, He Z (2009) Spermatogonial stem cells: mouse and human comparisons. Birth Defects Res C Embryo Today 87(1):27–34
Eddy EM (1975) Germ plasm and the differentiation of the germ cell line. Int Rev Cytol 43:229–280
Eddy EM, Hahnel AC (1983) Establishment of the germ cell line in mammals. In: McLaren A, Wylie CC (eds) Current problems in germ cell differentiation. Cambridge University Press, Cambridge, pp 41–69
Ehmcke J, Schlatt S (2006) A revised model for spermatogonial expansion in man: lessons from non-human primates. Reproduction 132(5):673–680
Ehmcke J, Wistuba J, Schlatt S (2006) Spermatogonial stem cells: questions, models and perspectives. Hum Reprod Update 12(3):275–282
El Ramy R, Verot A, Mazaud S et al (2005) Fibroblast growth factor (FGF) 2 and FGF9 mediate mesenchymal-epithelial interactions of peritubular and sertoli cells in the rat testis. J Endocrinol 187(1):135–147
Ellis SJ, Tanentzapf G (2010) Integrin-mediated adhesion and stem-cell-niche interactions. Cell Tissue Res 339(1):121–130
Fagoonee S, Hobbs RM, De Chiara L et al (2010) Generation of functional hepatocytes from mouse germ line cell-derived pluripotent stem cells in vitro. Stem Cells Dev 19(8):1183–1194
Falciatori I, Borsellino G, Haliassos N et al (2004) Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. FASEB J 18(2):376–378
Falender AE, Freiman RN, Geles KG et al (2005) Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID. Genes Dev 19(7):794–803
Feng LX, Ravindranath N, Dym M (2000) Stem cell factor/c-kit up-regulates cyclin D3 and promotes cell cycle progression via the phosphoinositide 3-kinase/p70 S6 kinase pathway in spermatogonia. J Biol Chem 275(33):25572–25576
Feng LX, Chen Y, Dettin L et al (2002) Generation and in vitro differentiation of a spermatogonial cell line. Science 297(5580):392–395
Fijak M, Bhushan S, Meinhardt A (2011) Immunoprivileged sites: the testis. Methods Mol Biol 677:459–470
França LR, Ogawa T, Avarbock MR et al (1998) Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biol Reprod 59(6):1371–1377
García-Castro MI, Anderson R, Heasman J et al (1997) Interactions between germ cells and extracellular matrix glycoproteins during migration and gonad assembly in the mouse embryo. J Cell Biol 138(2):471–480
Geens M, Goossens E, De Block G et al (2008) Autologous spermatogonial stem cell transplantation in man: current obstacles for a future clinical application. Hum Reprod Update 14(2):121–130
Giuili G, Tomljenovic A, Labrecque N et al (2002) Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. EMBO Rep 3(8):753–759
Goertz MJ, Wu Z, Gallardo TD et al (2011) Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. J Clin Invest 121(9):3456–3466
Golestaneh N, Beauchamp E, Fallen S et al (2009a) Wnt signaling promotes proliferation and stemness regulation of spermatogonial stem/progenitor cells. Reproduction 138(1):151–162
Golestaneh N, Kokkinaki M, Pant D et al (2009b) Pluripotent stem cells derived from adult human testes. Stem Cells Dev 18(8):1115–1126
Goossens E, Frederickx V, De Block G et al (2003) Reproductive capacity of sperm obtained after germ cell transplantation in a mouse model. Hum Reprod 18(9):1874–1880
Goossens E, Frederickx V, de Block G et al (2006) Evaluation of in vivo conception after testicular stem cell transplantation in a mouse model shows altered post-implantation development. Hum Reprod 21(8):2057–2060
Greenbaum MP, Yan W, Wu MH et al (2006) TEX14 is essential for intercellular bridges and fertility in male mice. Proc Natl Acad Sci USA 103(13):4982–4987
Griswold MD (ed) (2007) Perspective on the function of Sertoli cells. In: Sertoli cell biology. Elsevier, Amsterdam, pp 15–18

Gu Y, Runyan C, Shoemaker A et al (2009) Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration. Development 136(8):1295–1303
Guan K, Nayernia K, Maier LS et al (2006) Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 440(7088):1199–1203
Guan K, Wagner S, Unsold B et al (2007) Generation of functional cardiomyocytes from adult mouse spermatogonial stem cells. Circ Res 100(11):1615–1625
Guan K, Wolf F, Becker A et al (2009) Isolation and cultivation of stem cells from adult mouse testes. Nat Protoc 4(2):143–154
Guan K, Cheng IF, Baazm M (2012) Human spermatagonial stem cells: a novel therapeutic hope for cardiac regeneration and repair? Future Cardiol 8(1):39–51
Hamra FK, Gatlin J, Chapman KM et al (2002) Production of transgenic rats by lentiviral transduction of male germ-line stem cells. Proc Natl Acad Sci USA 99(23):14931–14936
Hamra FK, Schultz N, Chapman KM et al (2004) Defining the spermatogonial stem cell. Dev Biol 269(2):393–410
Hamra FK, Chapman KM, Wu Z et al (2008) Isolating highly pure rat spermatogonial stem cells in culture. Methods Mol Biol 450:163–179
He Z, Jiang J, Kokkinaki M et al (2008) Gdnf upregulates c-Fos transcription via the Ras/Erk1/2 pathway to promote mouse spermatogonial stem cell proliferation. Stem Cells 26(1):266–278
He Z, Kokkinaki M, Jiang J et al (2010) Isolation, characterization, and culture of human spermatogonia. Biol Reprod 82(2):363–372
He Z, Kokkinaki M, Jiang J et al (2012) Isolation of human male germ-line stem cells using enzymatic digestion and magnetic-activated cell sorting. Methods Mol Biol 825:45–57
Hermann BP, Sukhwani M, Lin C-C et al (2007) Characterization, cryopreservation, and ablation of spermatogonial stem cells in adult rhesus macaques. Stem Cells 25(9):2330–2338
Hermann BP, Sukhwani M, Hansel MC et al (2010) Spermatogonial stem cells in higher primates: are there differences from those in rodents? Reproduction 139(3):479–493
Hobbs RM, Seandel M, Falciatori I et al (2010) Plzf regulates germline progenitor self-renewal by opposing mTORC1. Cell 142(3):468–479
Hofmann M-C, Braydich-Stolle L, Dym M (2005) Isolation of male germ-line stem cells; influence of GDNF. Dev Biol 279(1):114–124
Huckins C (1971) The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat Rec 169(3):533–557
Iwasa T, Baba S, Doi H et al (2010) Neonatal mouse testis-derived multipotent germline stem cells improve the cardiac function of acute ischemic heart mouse model. Biochem Biophys Res Commun 400(1):27–33
Izadyar F, Pau F, Marh J et al (2008) Generation of multipotent cell lines from a distinct population of male germ line stem cells. Reproduction 135(6):771–784
Izadyar F, Wong J, Maki C et al (2011) Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. Hum Reprod 26(6):1296–1306
Jegou B (1992) The sertoli cell. Baillieres Clin Endocrinol Metab 6(2):273–311
Johnson L (1994) A new approach to study the architectural arrangement of spermatogenic stages revealed little evidence of a partial wave along the length of human seminiferous tubules. J Androl 15(5):435–441
Johnson L, Neaves WB, Barnard JJ et al (1999) A comparative morphological study of human germ cells in vitro or in situ within seminiferous tubules. Biol Reprod 61(4):927–934
Johnson L, Staub C, Neaves WB et al (2001) Live human germ cells in the context of their spermatogenic stages. Hum Reprod 16(8):1575–1582
Kanatsu-Shinohara M, Inoue K, Lee J et al (2004) Generation of pluripotent stem cells from neonatal mouse testis. Cell 119(7):1001–1012
Kanatsu-Shinohara M, Ikawa M, Takehashi M et al (2006) Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells. Proc Natl Acad Sci USA 103(21):8018–8023
Kanatsu-Shinohara M, Takehashi M, Takashima S et al (2008) Homing of mouse spermatogonial stem cells to germline niche depends on beta1-integrin. Cell Stem Cell 3(5):533–542

Kato Y, Kaneda M, Hata K et al (2007) Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. Hum Mol Genet 16(19):2272–2280

Kattman SJ, Huber TL, Keller GM (2006) Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. Dev Cell 11(5):723–732

Ko K, Tapia N, Wu G et al (2009) Induction of pluripotency in adult unipotent germline stem cells. Cell Stem Cell 5(1):87–96

Ko K, Arauzo-Bravo MJ, Tapia N et al (2010) Human adult germline stem cells in question. Nature 465(7301):E1; discussion E3

Kossack N, Meneses J, Shefi S et al (2009) Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells 27(1):138–149

Kubota H, Brinster RL (2006) Technology insight: in vitro culture of spermatogonial stem cells and their potential therapeutic uses. Nat Clin Pract Endocrinol Metab 2(2):99–108

Kubota H, Avarbock MR, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci USA 100(11):6487–6492

Kubota H, Avarbock MR, Brinster RL (2004a) Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 71(3):722–731

Kubota H, Avarbock MR, Brinster RL (2004b) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci USA 101(47):16489–16494

Kung JW, Forbes SJ (2009) Stem cells and liver repair. Curr Opin Biotechnol 20(5):568–574

Lassalle B, Bastos H, Louis JP et al (2004) 'Side Population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. Development 131(2):479–487

Lawson KA, Dunn NR, Roelen BA et al (1999) Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev 13(4):424–436

Lee J, Kanatsu-Shinohara M, Inoue K et al (2007) Akt mediates self-renewal division of mouse spermatogonial stem cells. Development 134(10):1853–1859

Lim JJ, Sung SY, Kim HJ et al (2010) Long-term proliferation and characterization of human spermatogonial stem cells obtained from obstructive and non-obstructive azoospermia under exogenous feeder-free culture conditions. Cell Prolif 43(4):405–417

Looijenga LH, Stoop H, de Leeuw HP et al (2003) POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. Cancer Res 63(9):2244–2250

Mahowald AP, Hennen S (1971) Ultrastructure of the "germ plasm" in eggs and embryos of Rana pipiens. Dev Biol 24(1):37–53

Mark M, Jacobs H, Oulad-Abdelghani M et al (2008) STRA8-deficient spermatocytes initiate, but fail to complete, meiosis and undergo premature charomosome condensation. J Cell Sci 121(19):3233–3242

Matsui Y, Zsebo K, Hogan BL (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70(5):841–847

Matzuk MM, Lamb DJ (2008) The biology of infertility: research advances and clinical challenges. Nat Med 14(11):1197–1213

McLaren A (2003) Primordial germ cells in the mouse. Dev Biol 262(1):1–15

Meistrich ML (1993) Effects of chemotherapy and radiotherapy on spermatogenesis. Eur Urol 23(1):136–141; discussion 142

Meng X, Lindahl M, Hyvonen ME et al (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287(5457):1489–1493

Mizrak SC, Chikhovskaya JV, Sadri-Ardekani H et al (2010) Embryonic stem cell-like cells derived from adult human testis. Hum Reprod 25(1):158–167

Nagano M, Patrizio P, Brinster RL (2002) Long-term survival of human spermatogonial stem cells in mouse testes. Fertil Steril 78(6):1225–1233

Nakagawa T, Sharma M, Nabeshima Y et al (2010) Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 328(5974):62–67
Naughton CK, Jain S, Strickland AM et al (2006) Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol Reprod 74(2):314–321
Oakberg EF (1971) Spermatogonial stem-cell renewal in the mouse. Anat Rec 169(3):515–531
Oatley JM, Brinster RL (2008) Regulation of spermatogonial stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 24:263–286
Oatley JM, Avarbock MR, Telaranta AI et al (2006) Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc Natl Acad Sci USA 103(25):9524–9529
Oatley JM, Avarbock MR, Brinster RL (2007) Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. J Biol Chem 282(35):25842–25851
Ogawa T, Dobrinski I, Avarbock MR et al (1998) Leuprolide, a gonadotropin-releasing hormone agonist, enhances colonization after spermatogonial transplantation into mouse testes. Tissue Cell 30(5):583–588
Ohbo K, Yoshida S, Ohmura M et al (2003) Identification and characterization of stem cells in prepubertal spermatogenesis in mice. Dev Biol 258(1):209–225
Ohinata Y, Payer B, O'Carroll D et al (2005) Blimp1 is a critical determinant of the germ cell lineage in mice. Nature 436(7048):207–213
Ohmura M, Yoshida S, Ide Y et al (2004) Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. Arch Histol Cytol 67(4):285–296
Orwig KE, Ryu BY, Master SR et al (2008) Genes involved in post-transcriptional regulation are overrepresented in stem/progenitor spermatogonia of cryptorchid mouse testes. Stem Cells 26(4):927–938
Resnick JL, Bixler LS, Cheng L et al (1992) Long-term proliferation of mouse primordial germ cells in culture. Nature 359(6395):550–551
Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. Nature 434(7035):843–850
Rideout WM 3rd, Hochedlinger K, Kyba M et al (2002) Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell 109(1):17–27
Ronaghi M, Erceg S, Moreno-Manzano V et al (2010) Challenges of stem cell therapy for spinal cord injury: human embryonic stem cells, endogenous neural stem cells, or induced pluripotent stem cells? Stem Cells 28(1):93–99
Ryu B-Y, Orwig KE, Kubota H et al (2004) Phenotypic and functional characteristics of spermatogonial stem cells in rats. Dev Biol 274(1):158–170
Ryu BY, Orwig KE, Oatley JM et al (2006) Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. Stem Cells 24(6):1505–1511
Sadri-Ardekani H, Mizrak SC, van Daalen SK et al (2009) Propagation of human spermatogonial stem cells in vitro. JAMA 302(19):2127–2134
Saitou M, Barton SC, Surani MA (2002) A molecular programme for the specification of germ cell fate in mice. Nature 418(6895):293–300
Saitou M, Kagiwada S, Kurimoto K (2012) Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. Development 139(1):15–31
Salih DAM, Brunet A (2008) FoxO transcription factors in the maintenance of cellular homeostasis during aging. Curr Opin Cell Biol 20(2):126–136
Sato M, Kimura T, Kurokawa K et al (2002) Identification of PGC7, a new gene expressed specifically in preimplantation embryos and germ cells. Mech Dev 113(1):91–94
Schaefer CB, Ooi SK, Bestor TH et al (2007) Epigenetic decisions in mammalian germ cells. Science 316(5823):398–399
Seandel M, James D, Shmelkov SV et al (2007) Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature 449(7160):346–350
Seki Y, Yamaji M, Yabuta Y et al (2007) Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. Development 134(14):2627–2638

Shamblott MJ, Axelman J, Wang S et al (1998) Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci USA 95(23):13726–13731
Shetty G, Meistrich ML (2005) Hormonal approaches to preservation and restoration of male fertility after cancer treatment. J Natl Cancer Inst Monogr 34:36–39
Shiba Y, Hauch KD, Laflamme MA (2009) Cardiac applications for human pluripotent stem cells. Curr Pharm Des 15(24):2791–2806
Shima JE, McLean DJ, McCarrey JR et al (2004) The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod 71(1):319–330
Shinohara T, Avarbock MR, Brinster RL (1999) Beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci USA 96(10):5504–5509
Simon L, Ekman GC, Garcia T et al (2010) ETV5 regulates sertoli cell chemokines involved in mouse stem/progenitor spermatogonia maintenance. Stem Cells 28(10):1882–1892
Small CL, Shima JE, Uzumcu M et al (2005) Profiling gene expression during the differentiation and development of the murine embryonic gonad. Biol Reprod 72(2):492–501
Spradling A, Drummond-Barbosa D, Kai T (2001) Stem cells find their niche. Nature 414(6859):98–104
Streckfuss-Bomeke K, Vlasov A, Hulsmann S et al (2009) Generation of functional neurons and glia from multipotent adult mouse germ-line stem cells. Stem Cell Res 2(2):139–154
Strome S, Lehmann R (2007) Germ versus soma decisions: lessons from flies and worms. Science 316(5823):392–393
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
Tapia N, Arauzo-Bravo MJ, Ko K et al (2011) Concise review: challenging the pluripotency of human testis-derived ESC-like cells. Stem Cells 29(8):1165–1169
Tegelenbosch RA, de Rooij DG (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 290(2):193–200
Tokuda M, Kadokawa Y, Kurahashi H et al (2007) CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol Reprod 76(1):130–141
Tolkunova EN, Malashicheva AB, Chikhirzhina EV et al (2009) E-cadherin as a novel surface marker of spermatogonial stem cells. Tsitologiia 51(3):212–218
Turovets N, Semechkin A, Kuzmichev L et al (2011) Derivation of human parthenogenetic stem cell lines. Methods Mol Biol 767:37–54
Tyagi G, Carnes K, Morrow C et al (2009) Loss of Etv5 decreases proliferation and RET levels in neonatal mouse testicular germ cells and causes an abnormal first wave of spermatogenesis. Biol Reprod 81(2):258–266
Van Pelt AM, De Rooij DG (1990a) The origin of the synchronization of the seminiferous epithelium in vitamin A-deficient rats after vitamin A replacement. Biol Reprod 42(4):677–682
Van Pelt AM, de Rooij DG (1990b) Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. Biol Reprod 43(3):363–367
Varanou A, Page CP, Minger SL (2008) Human embryonic stem cells and lung regeneration. Br J Pharmacol 155(3):316–325
Vergouwen RP, Jacobs SG, Huiskamp R et al (1991) Proliferative activity of gonocytes, sertoli cells and interstitial cells during testicular development in mice. J Reprod Fertil 93(1):233–243
Verhoeven G, Hoeben E, De Gendt K (2000) Peritubular cell-sertoli cell interactions: factors involved in PmodS activity. Andrologia 32(1):42–45
Wu X, Schmidt JA, Avarbock MR et al (2009a) Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. Proc Natl Acad Sci USA 106(51):21672–21677
Wu Z, Falciatori I, Molyneux LA et al (2009b) Spermatogonial culture medium: an effective and efficient nutrient mixture for culturing rat spermatogonial stem cells. Biol Reprod 81(1):77–86

Wu Z, Luby-Phelps K, Bugde A et al (2009c) Capacity for stochastic self-renewal and differentiation in mammalian spermatogonial stem cells. J Cell Biol 187(4):513–524

Yabuta Y, Kurimoto K, Ohinata Y et al (2006) Gene expression dynamics during germline specification in mice identified by quantitative single-cell gene expression profiling. Biol Reprod 75(5):705–716

Yang L, Soonpaa MH, Adler ED et al (2008) Human cardiovascular progenitor cells develop from a KDR + embryonic-stem-cell-derived population. Nature 453(7194):524–528

Yeh JR, Zhang X, Nagano MC (2011) Wnt5a is a cell-extrinsic factor that supports self-renewal of mouse spermatogonial stem cells. J Cell Sci 124(Pt 14):2357–2366

Yoshida S, Nabeshima Y, Nakagawa T (2007a) Stem cell heterogeneity: actual and potential stem cell compartments in mouse spermatogenesis. Ann N Y Acad Sci 1120:47–58

Yoshida S, Sukeno M, Nabeshima Y-I (2007b) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317(5845):1722–1726

Yoshinaga K, Nishikawa S, Ogawa M et al (1991) Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. Development 113(2):689–699

Zheng K, Wu X, Kaestner KH et al (2009) The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol 9:38

Zhou Q, Li Y, Nie R et al (2008a) Expression of stimulated by retinoic acid gene 8 (Stra8) and maturation of murine gonocytes and spermatogonia induced by retinoic acid in vitro. Biol Reprod 78(3):537–545

Zhou Q, Nie R, Li Y et al (2008b) Expression of stimulated by retinoic acid gene 8 (Stra8) in spermatogenic cells induced by retinoic acid: an in vivo study in vitamin A-sufficient postnatal murine testes. Biol Reprod 79(1):35–42

Zhu S, Wei W, Ding S (2011) Chemical strategies for stem cell biology and regenerative medicine. Annu Rev Biomed Eng 13:73–90

Chapter 10
Hematopoietic Stem Cells

Mary L. Clarke and Jonathan Frampton

Abstract Hematopoietic stem cells represent the most studied and understood adult stem cell, and have consequently set the trends for the investigation of a wide array of stem cells, while their clinical use for over half a century and ever improving efficacy encourages the view that stem cell therapy will one day be useful in the treatment of a whole host of diseases that involve cellular loss. In this chapter we describe how hematopoietic stem cells can be identified, isolated and characterized, and how important it is to be able to conduct experiments on animal models as well as humans, especially as studies in animals can provide the best, sometimes only, way to test stem cell potential and new protocols for their therapeutic use. The increasing possibilities for bone marrow regenerative medicine raised by the rapid developments in our ability to derive pluripotent stem cells from any individual are discussed, in particular because these are likely to be a very effective source of hematopoietic stem cells for all people requiring them to be replaced, as well as the exciting prospect that they can provide a route for the correction of inherited diseases affecting the blood system.

10.1 Introduction

The hematopoietic stem cell (HSC) represents a paradigm for much of present day stem cell biology and regenerative medicine, the first therapeutic application, predating any knowledge of its characterization or even of its actual existence, being the pioneering development by E. Donnall Thomas in 1957 of bone marrow transplantation

M.L. Clarke, Ph.D. • J. Frampton, B.A., Ph.D. (✉)
Institute of Biomedical Research, College of Medicine and Dental Science,
University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
e-mail: J.Frampton@bham.ac.uk

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_10,

(BMT) as a therapy to alleviate the consequences of radiation and chemotherapy (Thomas et al. 1957). This groundbreaking therapy formed the dogma that tissue stem cells held the future promise for regenerative medicine for numerous diseases. The strategies for characterization, purification and bioassay of HSCs have therefore been adapted for many other tissue-specific stem cells, while the drive to understand the cellular and molecular properties of HSCs has provided a framework for comparison to both embryonic and adult stem cell types. Studies on HSCs and comparison to the behaviour of leukemia cells was also highly influential in the origin of the concept of the cancer stem cell as the underlying component of many, perhaps all, tumors, and an exciting target for novel therapies that may succeed in achieving life-long remission where traditional treatments often fail.

Given the extensive history in the use of HSCs in therapeutic practice, it would be easy to assume that these cells are well understood, however HSC research is a dynamic area, continually being revolutionized. Once believed to be a homogenous population, it has since emerged that the HSCs are actually not a single entity, but rather a collection of cell subtypes with largely pre-programmed differentiation and self-renewal behaviours, both of which will be discussed in detail below. The nature of HSCs also varies throughout development, distinct cell types arising to provide a transient source of hematopoietic cells. Since it is beyond the scope of this chapter, the reader is referred to one of the many excellent reviews that discuss the developmental aspects of HSC biology (Dzierzak et al. 1998; Mikkola and Orkin 2006; Cumano and Godin 2007; Dzierzak and Speck 2008), and here we will concentrate on those HSCs that have the most relevance to regenerative medicine, namely adult bone marrow derived cells and those HSCs that can be isolated from umbilical cord blood.

The necessity for continual regeneration of the various lymphoid (B-cells, T-cells, natural killer cells, dendritic cells) and myeloid (red cells, platelets, monocytes/macrophage, dendritic cells, granulocytes) cells that constitute the hematopoietic system is emphasized when we consider the vast number of cells, approximately 10^{12}, arising in human bone marrow on a daily basis (Doulatov et al. 2012). These mature adult hematopoietic cells are generated through a succession of hierarchical steps initiating at the apex of the hematopoietic system with the HSC. The HSC gives rise to a series of transient amplifying progenitor cell populations with a gradual decrease in proliferative potential and an increase in cellular specialization, resulting ultimately in the supply of terminally differentiated functional blood cell types that make up the lymphoid and myeloid compartments. Although the hematopoietic system has been extensively studied for several decades, it is only recently that we have begun to understand some of the mechanisms by which HSCs are able to so proficiently play their role. These developments have been made with the help of improving technology, allowing complex cell sorting strategies to isolate rare HSCs to high purity and viability in order to further quantify and characterise them.

Parallel studies are now being done with human HSCs but the advancement of our knowledge of these cells is trailing behind that of the mouse. The primary indicator of stem cell activity being their ability to function in repopulation assays poses an obvious difficulty in human stem cell research. Secondly, a major obstacle in

human HSC research is that the cells are incredibly rare, with only 1 in 10^6 cells in human bone marrow being a functional transplantable stem cell (Wang et al. 1997) and the availability of novel markers of these cells for purification from the bulk of differentiated cells hinders progress further.

In addition to increasingly refined definition of HSCs, especially those that have the greatest potential for application in a therapeutic context, key areas of investigation that will impinge heavily on the success or otherwise of advances in regenerative medicine include finding ways to expand HSCs in vitro without loss of any aspect of their functional potential, and improving upon the efficiency with which transplanted cells integrate into the hematopoietic system. However, perhaps the most exciting challenge, which could 1 day lead to an unlimited ability to provide replacement HSCs personalised for the patient, is their derivation from pluripotent stem cells, and this will be discussed with respect to advances that have been made using embryonic stem (ES) cells and more recently with the discovery of methods to produce so called induced pluripotent stem (iPS) cells from any cell in the body.

10.2 Derivation/Classification

Although the first application of bone marrow derived stem cells in a therapeutic context occurred over five decades ago, the vast majority of our understanding of the nature of HSCs has come from studies on mouse bone marrow. The single biggest hurdle in the identification and purification of HSCs from mouse bone marrow is their very low abundance; depending on the precise criteria applied this is only 0.05% or less of the nucleated cells, resulting in the isolation of around 5,000 HSCs per mouse. Modern day laboratories utilize two main methods for isolating HSCs from bone marrow. First is an enrichment method (MACS) employing magnetic beads conjugated to antibodies against a specific surface marker. The second, and notably more precise separation technique, utilizes fluorescence activated cell sorting (FACS), which is based on immunofluorescent labeling of surface antigens as an analytical tool to achieve cell sorting (Challen et al. 2009). Modern cell sorters are now equipped to analyse up to 18 fluorochrome-labeled antibodies directed against multiple markers (usually designated as 'Cluster of Differentiation' or CD markers) enabling prospective isolation of more infrequent cells, which can then be subjected to bioassay to assess their stem cell potency.

10.2.1 Bioassays of HSCs

In the strictest sense, the HSC is defined by its functional capacity to reconstitute the entire hematopoietic system for the lifetime of the individual or animal; however, a number of less stringent bioassays are also widely used, often as a preliminary guide because the definitive in vivo bone marrow transplantation assay is both time-consuming and costly.

The first assays of hematopoietic progenitor cell potential in vivo can be attributed to James Till and Ernest McCulloch, who famously demonstrated that colonies of myeloid cells developing in the spleen following transplantation of bone marrow into lethally irradiated mice were clonal (Till and McCulloch 1961). However, the existence of long-lived stem cells in the bone marrow was deduced from subsequent experiments involving clonal tracking of serial transplantations (Dick et al. 1985; Lemischka et al. 1986). Arising out of these studies, the current gold standard assay is generally accepted to be long-term repopulation of lethally irradiated mice in a situation in which the cells being tested are compared to a reference wild type population (Harrison et al. 1993), most often using test and reference strains that are congenic for allelic variants of CD45 (previously known as Ly5), which can easily be distinguished by immunofluorescent flow cytometry. 'Long-term' is taken to mean a sustained output from the graft of at least 1% of all circulating white blood cells for at least 4 months (Purton and Scadden 2007), but the most rigorous test of HSC potential involves assessment of their ability to be serially transplanted from the primary reconstituted recipient to a secondary irradiated host, thereby demonstrating that engrafting cells are undergoing self-renewal. Competitive repopulation assays performed this way are at best semi-quantitative, and a more refined method, involving limiting dilution, allows determination of the frequency of HSCs. In this assay, a series of dilutions of the test population are competed against reference wild type bone marrow cells. The number of mice negative for reconstitution in each cell dose is measured and the frequency of HSCs ('competitive repopulating units' or CRU) is estimated using Poisson statistics (Szilvassy et al. 1990). Purton and Scadden (2007) discuss the finer details of repopulation assays, how they are best interpreted, and their possible limitations.

Although in vivo assays are essential in order to fully define and quantify stem cell potential, they have some limitations that can be complemented by a range of assays that can be performed in vitro. First, and rather obviously, in vivo assays can take many months to complete and require extensive and costly facilities, so it is often useful to have a more simple assay that can be used to make an initial assessment of the likely HSC content, for example while developing a strategy for prospective cell sorting or following some experimental manipulation that is expected to have a significant effect on HSC function. Second, the output from an in vivo assay is the consequence of many biological events following transplantation, including homing, self-renewal, HSC commitment and the behaviour of downstream progenitors and differentiated cells, and it is often important to be able to determine cellular properties at a single cell level immediately following isolation of putative HSCs. Several distinct in vitro assays are used that measure the frequency of progenitors (colony-forming unit in culture; CFU-C), stem cells (long-term culture-initiating cell; LTC-IC), or both (cobblestone area-forming cell assay; CAFC), the latter two correlating at least to some extent with in vivo activity (van Os et al. 2004).

CFU-C assays, pioneered by the work of Don Metcalf and colleagues (Bradley and Metcalf 1966; Moore et al. 1973), allow detection and quantification of myeloid progenitors present in the population of cells being analyzed or that could have arisen in vitro from more immature cells, including the HSC. The culture conditions rely on the presence of growth factors and nutrients that will permit complete differentiation

along one or more of the pathways of differentiation that a particular cell is expected to be capable of adopting. CFU-C assays have been essential for determining the specific growth factors necessary for HSC maintenance, proliferation and differentiation. They have also been crucial in the characterisation of leukemic stem cells (LSCs). The assessment of lymphoid CFU potential in vitro has in the past been more difficult, requiring co-culture systems such as that of OP9-DL1 cells, a mouse stromal cell line that ecotropically expresses the Notch ligand Delta-like 1 (DL1) for establishing T-cell differentiation (Whitlock and Witte 1982; Schmitt and Zúñiga-Pflücker 2002). However, recent demand for improved mouse B-cell differentiation has led to the development of media capable of supporting such specification, similar to that already used for myeloid lineages. The progress for human lymphoid cell differentiation is, however, still somewhat marred due to the insufficient knowledge of the cytokines responsible for this (Doulatov et al. 2012). Human B-cell differentiation is feasible when HSCs are co-cultured for 2–4 weeks upon the stromal cell lines MS-5 or S17 in the presence of SCF, TPO, IL-7 and IL-2.

The basic principle of such in vitro assays is to determine what a stem cell or progenitor is capable of giving rise to and their proliferative abilities following gene manipulation, as can be recognized after a number of days by the specific features of the differentiated cells (for example surface marker expression, cell morphology and the presence of characteristic cytoplasmic enzyme activities, etc). In the right conditions, a HSC can give rise to multiple cell lineages, whereas a more mature hematopoietic progenitor cell will have a more restricted capability. Since it would not be possible to discriminate from whence the individual differentiated cells originated if such assays were performed in a liquid culture of the whole sorted population, these assays are generally carried out in one of two ways so that the potential of individual cells can be observed. Most commonly, a cell population is seeded into the appropriate growth conditions in media that also contain a substance that is like a soft gel (usually methycellulose). This prevents the cells from moving around extensively, and if seeded at the correct density means that the differentiated derivatives from each cell are clearly separated and can eventually be collected for phenotypic analysis. Alternatively, sorted stem cells can be deposited as single cells into tiny individual wells in a plastic dish where they then can be allowed to grow and differentiate in liquid conditions (Ema et al. 2000; Takano et al. 2004).

The CAFC and LTC-IC assays, based on the original studies by Dexter and colleagues (Dexter et al. 1977), involve culture of stem cell populations with adherent cells that mimic the normal HSC microenvironment. The CAFC assay measures the frequency of cells that are capable of growing under the stromal layer, and by enumerating so-called ‘cobblestone’ areas at various times it is possible to assess mature progenitors back to repopulating HSCs (Ploemacher et al. 1989, 1991). The LTC-IC assay is similar to the CAFC assay except that the readout is the presence of progenitors that can themselves be assayed for CFU-C capability (Sutherland et al. 1989; Lemieux et al. 1995).

Just as many of the in vitro assays are adaptable for the measurement of both murine and human stem cells and progenitors, there is an equal and ever growing need in the context both of regenerative medicine and for therapeutic targeting of diseased cells to be able to test in vivo potential of human HSCs. Since this is clearly

not feasible in humans a number of approaches have been developed over the last 30 years that rely on the generation of in vivo chimeras of human cells, or 'xenografts', in animals. Although not ideal from the perspective of logistics, cost, and time, several investigators have shown that human HSCs can be engrafted in sheep by direct introduction of cells into the early gestational age fetus. By in utero injection of human fetal liver HSCs, Esmail Zanjani and colleagues were able to demonstrate long-term (greater than 2 years) engraftment in sheep (Zanjani et al. 1992). Subsequently, this approach was used to prove for the first time that human adult bone marrow cells could elicit long-term chimerism and sustain human hematopoiesis (Srour et al. 1993), and has been further developed (Zanjani et al. 1995, 1998), enabling most recently the demonstration that engrafted human cells can be mobilized in the sheep model in the same way that they would normally be in human donors (Almeida-Porada et al. 2007).

By far the majority of xenograft experiments of human HSCs have been performed in mice, but unlike the experiments involving sheep, the human cells are injected into adults, thereby requiring that the hosts are immunologically deficient so that they will not bring about rejection of the xenogenic cells. Two strategies for human-into-mouse engraftment were developed originally by the work of McCune and Dick. In the first approach, human cells within a fragment of hematopoietic tissue are grafted under the kidney capsule, which provides a permissive environment for the donor cells (McCune et al. 1988; Namikawa et al. 1990; Chen et al. 1994). However, the much more widely utilized method involves adaptation of the usual protocol for transplantation of mouse HSCs into host animals, the main difference being that the mice are subjected to sub-lethal irradiation to pre-condition the bone marrow by increasing the opportunity for HSCs to occupy vacant niches (Kamel-Reid and Dick 1988).

To minimize the possibility of the human cells being rejected, several immunocompromised mouse models have been developed, in particular relying upon the Severe Combined Immunodeficiency (SCID) mutant strain (for reviews see Greiner et al. 1998; Pearson et al. 2008). As for mouse-into-mouse repopulation assays, xenografts of human cells can be made quantitative through a limiting dilution approach, the term SCID Repopulating Unit (SRU) usually being adopted to define the numbers of functional long-term HSCs. SCID mice are deficient in both B- and T-cell mediated immunity, and their usefulness has been enhanced in various ways through combination with other spontaneous or engineered mutations. The strain that has been most commonly used for xenografting is a combination of the Non-Obese Diabetic (NOD) mutation with SCID, usually referred to as NOD/SCID, which lacks not only functional B and T lymphocytes, but also has low levels of natural killer (NK) cell activity (Shultz et al. 1995). A reduced overall cellularity of the bone marrow in NOD/SCID mice may also facilitate engraftment of HSCs because of the availability of suitable niches for stem cells. The NOD/SCID mouse exhibits some features that partially limits its usefulness, especially for long-term xenograft models, such as shortened lifespan due to high incidence of thymic lymphoma, some spontaneous production of functional lymphocytes with aging, and residual innate immunity. Further incorporation of the β2 microglobulin knockout

into the NOD/SCID background increased the efficiency of repopulation by umbilical cord blood cells by over tenfold (Kollet et al. 2000).

To circumvent the problems associated with NOD/SCID mice as models for xenografts, NOD/SCID mice with a truncation or a deletion of the IL-2R common γ chain, which is a critical component for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 signaling, were developed (the so-called 'NSG' strain, Ito et al. 2002). The deletion of the IL-2R common γ chain gene in mice results in a complete loss of B, T and NK cells. The NSG strain was shown to support a fivefold higher $CD34^+$ cell engraftment compared with NOD/SCID mice (Goldman et al. 1998). The second benefit of these mice is that the deficiency in cytokine signaling prevented the formation of lymphomas, permitting long-term studies (Ito et al. 2002; Ishikawa et al. 2005; reviewed by Ito et al. 2008).

As hematopoietic research progresses and the importance of specific growth factors are determined, many investigators have found that the efficiency of engraftment of human HSCs can be enhanced by co-transplantation of accessory cells or treatment of the host with cytokines. For example, Bonnet et al. (1999) demonstrated that low numbers of purified cord blood-derived immature cells would engraft NOD/SCID mice effectively if co-transplanted with more mature cell populations that had been irradiated to prevent cell division or by short-term in vivo treatment with the growth factors and cytokines stem cell factor (SCF), interleukin 3 (IL3) and granulocyte macrophage colony stimulating factor (GM-CSF). Similarly, bone marrow chimerism in the SCID or Rag2/IL2Rγ double knockout models could be facilitated by administration of IL3, GM-CSF and erythropoietin (Lapidot et al. 1992) or IL3, GM-CSF and erythropoietin (Mazurier et al. 1999), respectively. Co-transplantation of stromal cells has also been shown to have some benefit in establishing HSC xenografts in mice. Hence, primary bone marrow stroma modified to express IL3 was able to enhance HSC engraftment (Nolta et al. 1994), while unmodified mesenchymal stem cells (MSCs) derived from the fetal lung or bone marrow increased HSC engraftment, but in the latter case it appeared that the effect might not to require homing of MSC to the bone marrow (Noort et al. 2002; in 't Anker et al. 2003; Bensidhoum et al. 2004). Advances in the last year in strains for xenografting have seen the generation of mice with cytokine-expressing transgene knock-ins, encoding for example TPO, IL-3, and GM-CSF, all of which have exhibited augmented engraftment of the human cells following transplantation (Rongvaux et al. 2011; Willinger et al. 2011).

This xenograft method has also been adopted to assess LSC behaviour from human patient samples and can hence act as a model for therapeutic approaches.

The protocols for engraftment of HSCs have undergone a number of modifications over the years, including additional preconditioning of mice by treatment with clodronate-containing liposomes in order to delete macrophage (Fraser et al. 1995; van Rijn et al. 2003) or with an antibody against the surface antigen CD122 in order to target NK cells and macrophage that act as a barrier to stem cell engraftment (McKenzie et al. 2005). Furthermore, to overcome the limitations of homing and cellular loss in the lungs that is inherent in intravenous injection of cells via the tail vein, a number of investigators have achieved much improved rates of engraftment by direct injection of HSCs into the bone marrow cavities of either the femur or tibia (Kushida et al. 2001; Wang et al. 2003; Yahata et al. 2003; McKenzie et al. 2006).

10.2.2 HSC Antigenic Phenotype and Purification Schemes

Over the last quarter of a century the combined power of flow cytometry and the availability of monoclonal antibodies raised against a vast array of hematopoietic cell surface molecules, together with the various bioassays described above, has enabled an incredibly detailed definition of the heterogenous population of cells with stem cell activity in the hematopoietic hierarchy. With perhaps one exception that will be described later, no single surface molecule has yet been found that enables identification of HSCs; however, a number of markers have been described that can be used in combination to very precisely define stem cells, the particular drive being to isolate the rare cells with the highest potential for long-term reconstitution. The advances in this area have been most successful in the case of mouse bone marrow, and the current state of play will be elaborated before considering what we know about the phenotype of human HSCs derived from the bone marrow or umbilical cord blood.

10.2.2.1 Mouse HSCs

Most sorting strategies rely upon negative selection for markers of the differentiated hematopoietic lineages (Lin), which usually include B220, CD4 (sometimes CD3 or CD5 instead), CD8, Gr1, CD11b (Mac-1) and Ter119, in combination with positive selection for c-Kit (the receptor for SCF) and Sca-1 (stem cell antigen-1) (Okada et al. 1992), giving rise to the acronym LSK (or KSL, depending on laboratory preference). Historically, the Weissman group has been the driving force for the purification of HSCs and their favoured protocol incorporates staining for the Thy1.1 antigen and selection of cells that express only low levels together with an absence of lineage markers and the presence of Sca-1 ($Thy1.1^{lo}$ Lin^- $Sca\text{-}1^+$ or TLS cells; Spangrude et al. 1988), although this precise strategy has not been widely adopted because the Thy1.1 antigen is not expressed on many of the most commonly used laboratory strains of mice. Both LSK and TLS populations contain long-term repopulating cells (LT-HSCs), but these represent less than 10% of the LSK cells, the remains of which have only short-term activity (ST-HSCs) or are multipotent progenitors (MPPs) with no capacity for self-renewal. Following these early studies, there has been an ever-driving urge to discover a unique marker of the mouse LT-HSC that truly distinguishes it from the heterogeneous population of stem cells. A number of investigators have identified additional markers that can be used to resolve the stem cell and progenitor components within the LSK population, and to this day improvements are still being published on a fairly regular basis. The Nakauchi laboratory were first to show that the expression of CD34 could be used to discriminate LT-HSCs, in that single LSK $CD34^+$ cells were able to bring about long-term reconstitution (Osawa et al. 1996). The subsequent addition of Flt-3 (also known as Flk-2 and CD135) into the mix allowed prospective purification of not only LT-HSCs (LSK $CD34^-$ $Flt3^-$), but also ST-HSCs

(LSK CD34$^+$ Flt3$^-$) and MPPs (LSK CD34$^+$ Flt3$^+$) (Christensen and Weissman 2001; Adolfsson et al. 2001; Yang et al. 2005). More recently, due to the adoption of more precise cell sorting strategies, it has become possible to fractionate these stem cell populations further into more discrete fractions with more specific properties. Hence, data suggests that the MPP initially differentiates into lymphoid-primed multipotential progenitors (LMPPs), which retain the potential to give rise to lymphoid and granulocyte-macrophage cells but which lack megakaryocyte-erythroid potential (Adolfsson et al. 2005; Lai and Kondo 2006), while more committed myeloid and lymphoid progenitors lie downstream of these LMPP cells (Akashi et al. 2000; Pronk et al. 2007). These latter publications led to a redrawing of the accepted hematopoietic hierarchy model, and as further work elucidates more and more discrete sub-populations within the HSC compartment, it is likely that the hematopoietic hierarchy as it is currently understood will undergo yet more restructuring in the future.

In addition to surface marker expression, there are other characteristics of HSCs that can be employed for their identification and isolation using flow cytometry, often most effective when used in combination with strategies such as those employing LSK or related staining protocols. The most widely used characteristic relies on the ability of HSCs to actively expel small molecules from their cytoplasm, a mechanism of cytotoxic evasion. A family of transmembrane proteins known as ABC transporters are involved in a wide variety of normal cells and stem cells with the purpose of removing diverse chemicals. One family member, ABC-G2, is often expressed by stem cells and has the ability to export certain chemical dyes that have entered the cytoplasm by passive diffusion. Empirically, it was found that one such DNA binding supravital dye, Hoechst 33342, is removed by ABC-G2 and that this can be visualized with a flow cytometer by measuring red and blue fluorescent light emissions upon stimulating with a UV laser. In the complex pattern of light emitted by a mixture of cells treated with Hoechst 33342, many stem cells appear as a population, usually called the 'side population', which exhibits low red and blue fluorescence because the dye has been largely removed by the transporter (Goodell et al. 1996). The drawbacks of the Hoechst 33342 exclusion method are that the staining method is highly sensitive to slight changes in protocol, producing inconsistencies between HSC isolations. Unfortunately, side population characteristics are not restricted to stem cells, with approximately 15% of whole bone marrow side population being negative for the stem cell markers c-Kit and Sca-1 (Challen et al. 2009). Also, not all stem cells exhibit the property, and therefore the technique is best utilized in combination with other methods, especially surface marker staining, as a means to refine stem cell identification and isolation (Challen et al. 2009). The other major flow cytometry method not involving specific antibodies makes use of the fluorescent vital dye rhodamine 123 (Rh-123), which preferentially accumulates in mitochondrial membranes and acts as an indicator of mitochondrial, and hence cellular, activity. Since the more immature HSCs tend to be quiescent, sorting for cells exhibiting a low degree of fluorescence in the presence of Rh-123 enriches for long-term repopulating cells (Spangrude and Johnson 1990).

The most recent and highly resolved strategies for the isolation of long-term repopulating HSCs have largely built upon the basis of one or more of the LSK, side population and Rh-123 staining methods. Chen et al. (2003) found that immunofluorescent staining for the ancillary TGFβ receptor endoglin (CD105) in combination with Sca-1 expression and low staining for Rh-123 defines a nearly homogenous population of LT-HSCs without the use of CD34, c-Kit or Lin markers. As discussed later in this chapter, another feature of immature HSCs is their tendency to be niche-associated and a marker linked to this property, namely the angiopoietin-2 receptor Tie2, has been used to select a subpopulation of LSK cells that are enriched in LT-HSCs (Arai et al. 2004). Two advances based on RNA microarray screening for genes expressed exclusively in subfractions of HSCs have probably made the most significant contribution to the robust isolation of highly enriched long-term repopulating cells. First, following initial identification from expression screening, antibodies against cell surface receptors of the SLAM family, including CD48, CD150 and CD244, were shown to discriminate HSCs ($CD48^-$ $CD150^+$ $CD244^-$), MPPs ($CD48^-$ $CD150^-$ $CD244^+$) and the most restricted progenitors ($CD48^+$ $CD150^-$ $CD244^+$) (Kiel et al. 2005). This is the first family of receptors whose combinatorial expression can be used to precisely distinguish stem and progenitor cells in the mouse. Similarly, microarray technology led to the identification of murine endothelial protein C receptor (EPCR, CD201) as a marker to sort cells, especially when used in combination with positivity for the antigen Sca-1, as it is expressed at high levels in HSCs with a high reconstitution activity, and probably represents the first known marker that 'explicitly' identifies HSCs within murine bone marrow (Balazs et al. 2006). Most recently, the group of Conny Eaves has combined these two latter approaches and shown that LT-HSCs with the most durable self-renewal potential, as demonstrated following serial transplantation, are selectively and highly enriched in the $CD150^+$ subset of the $EPCR^+$ $CD48^-$ $CD45^+$ fraction of bone marrow cells (Kent et al. 2009).

10.2.2.2 Human HSCs

The ability to identify and purify long-term reconstituting human HSCs are at present somewhat less sophisticated compared to the situation with the mouse due to the lack of adequate methods to segregate HSCs from MPPs. Similar to the mouse, purification of human HSCs requires simultaneous detection of several cell surface markers, and although informative, the specific strategies for isolating mouse HSCs cannot be duplicated for human HSC. This is due to differences in characteristic marker expression between the two species, the most prominent difference residing in their expression of CD34. The two principal sources of human HSCs for therapeutic application, namely bone marrow and umbilical cord blood, also demonstrate some differences in the precise pattern of markers, raising extra difficulties in determining the best strategies for cell purification in the clinic. Nevertheless, human HSCs capable of multilineage engraftment in animal models can now be resolved with a reasonably high degree of enrichment.

The majority of human HSCs are $CD34^+$ in contrast to mouse, as was first demonstrated during the 1990s when human Lin^-CD34^+ fetal bone marrow cells were shown to be able to engraft in SCID mice (Baum et al. 1992). However, although capable of engrafting in SCID mice, most $CD34^+$ cells were subsequently shown to be lineage-restricted progenitors and the true HSC remained elusive. Enrichment of human HSCs can be achieved further on the basis of expression of CD45RA (Mayani et al. 1993), Thy-1 (Baum et al. 1992; Craig et al. 1993; Majeti et al. 2007) and CD38 (Hao et al. 1995; Bhatia et al. 1997). The recognized pattern of expression that segregates human HSCs from MPPs is that of $CD34^+$ $CD38^-CD45RA^-$ and loss of Thy1 expression (Majeti et al. 2007).

In contrast to $CD34^+$ subfractions, Lin^- $CD34^-$ $CD38^-$ cells have low clonogenicity in short-and long-term in vitro assays. However, the number of $CD34^-$ SRUs increased in short-term suspension cultures in conditions that did not maintain SRU derived from $CD34^+$ populations (Bhatia et al. 1998).

Based on its association with colony-forming potential and repopulation capacity, CD34 expression has, until recently, remained as a convenient marker for human HSCs. However, it has since been postulated that there is in fact a human $CD34^-$ HSC that is analogous to that of the mouse (Bhatia et al. 1998; Ando 2002; Engelhardt et al. 2002; Guo et al. 2003; Ishii et al. 2011), adding an increased complexity to the organisation of the human hematopoietic stem cell compartment.

Chimeras generated in either sheep or immunocompromised mice have shown that $CD34^-$ cells from cord blood, bone marrow, and granulocyte colony stimulating factor (G-CSF)-mobilized peripheral blood do have in vivo HSC activity in spite of failing to exhibit significant clonogenic activity in vitro. Further defining the phenotype of cord blood-derived $CD34^-$ SRUs, Kimura et al. (2007) proposed that the immunophenotype of very primitive long-term repopulating human HSCs is Lin^- $CD34^-$ c-Kit^- $Flt3^-$. Paralleling studies on mouse HSCs, Goodell et al. (1997) showed that human bone marrow contains side population cells and, interestingly, that these too are $CD34^-$. As in the mouse, Rh-123 staining can be employed in defining human HSCs, low dye retention being associated closely with the Lin^- $CD34^+$ $CD38^-$ population (McKenzie et al. 2007). However, taking all of this knowledge into account and using simple calculations of reported HSC frequencies, it can be established that more than 99% of human HSCs must be $CD34^+$.

The differences between antigen expression on mouse and human HSCs is not unique to CD34, and other distinctive variations can be seen in the expression of the Flt-3 receptor, which is expressed on the surface of human HSCs but not on the mouse (Sitnicka et al. 2003), and the SLAM marker CD150, which unlike in the mouse is absent on human HSCs (Sintes et al. 2008; Larochelle et al. 2011).

Due to the discrepancies in the expression of CD34 and its relationship to stem cell activity, research continues to define better markers of human HSCs. A recent publication from the laboratory of John Dick revealed a novel human HSC marker, namely CD49f (α6 integrin). Single $CD49f^+$ cells were shown to be capable of generating highly efficient long-term multilineage grafts and that loss of CD49f expression coincided with transient engrafting MPPs (Notta et al. 2011). Such markers could pave the way for the isolation of pure populations of human HSCs for therapeutic use and further research into HSC properties.

A number of additional discriminators of human HSC subpopulations have been investigated. Two features of human HSCs that have proven useful for the isolation of the most immature cells are worthy of mention. First, is the relative high expression of aldehyde dehydrogenase (ALDH) in hematopoietic progenitor cells (Kastan et al. 1990). Cord blood cells stained for ALDH activity using the substrate BODIPY-aminoacetaldehyde ('Aldefluor') and depleted for Lin^+ cells are enriched for $CD34^+$ $CD38^-$ cells (Storms et al. 1999). Second, and perhaps the more useful property of long-term repopulating human HSCs, is their expression of CD133, an antigen that characterizes several types of adult stem cells. For example, a rare population of cord blood cells expressing CD133 and negative for CD7 were found to be highly enriched for progenitor activity at a frequency equivalent to purified fractions of $CD34^+$ stem cells, and they were the only subset among the Lin^- $CD34^-$ $CD38^-$ population capable of giving rise to $CD34^+$ cells in defined liquid cultures and of engrafting in NOD/SCID mice (Yin et al. 1997; Gallacher et al. 2000). Cell selection combining Lin antigen depletion together with staining for ALDH activity and CD133 expression provides a purification of HSCs with long-term repopulating function that has been considered to be an alternative to CD34 cell selection for stem cell therapies. Hence, limiting dilution analysis demonstrates a tenfold increase in the frequency of repopulating cells compared with Lin^- $CD133^+$ cells, with maintenance of immature hematopoietic phenotypes ($CD34^+$ $CD38^-$) and enhanced repopulating function in recipients of serial, secondary transplants (Hess et al. 2006).

10.3 Characteristics/Properties

Like other adult stem cells, HSCs are regulated and supported by the surrounding tissue microenvironment, generally referred to as the stem cell 'niche'. As already discussed in detail in Chap. 3, the niche includes all cellular and non-cellular components that interact in order to control the adult stem cell, and the reader is also referred to a number of excellent recent reviews that specifically discuss the nature of these in relation to the HSC in the bone marrow (Taichman 2005; Wilson and Trumpp 2006; Li and Li 2006; Kiel and Morrison 2008; Raaijmakers and Scadden 2008; Mercier et al. 2011).

In brief, the current perception of HSCs in the bone marrow is that they reside at the interface of bone and the bone marrow (the endosteum), but it remains uncertain whether this interface itself is a niche, or whether endosteal cells secrete factors that diffuse to nearby niches. Indeed, recent work from the laboratory of David Scadden has shown that HSCs can reside in a niche that appears to involve a very close juxtaposition of both osteoblasts and microvessel endothelial cells (Lo Celso et al. 2009). Vascular or perivascular cells may also create niches as many HSCs are observed around sinusoidal blood vessels, and perivascular cells secrete factors that regulate HSC maintenance.

It is important that the bone marrow niche should not be viewed as a static environment, since both the hematopoietic and immune systems are required to respond rapidly and adapt to the needs of the individual, it should therefore be regarded as a

fluid system that continually processes information from the organism as a whole (Mercier et al. 2011). Much of the knowledge that we have obtained from studies of the normal bone marrow microenvironment is leading us to a better understanding of the ways in which leukemic stem cells (LSCs) can manipulate the niche to enhance their survival and proliferation. Due to this adaptable nature of the bone marrow niche it is becoming clear that it represents a novel therapeutic target, its manipulation, for example by pharmacological enhancement of the number and function of osteoblasts, being a potential way to augment the effectiveness of stem cell therapies (Adams and Scadden 2008).

Much of the life of a HSC within its niche is one of inactivity in which it replicates only relatively infrequently. This state of 'quiescence' is thought to be an indispensable property for the maintenance of HSCs, protecting them from stress and hence the accumulation of DNA mutations and enabling them to sustain lifelong hematopoiesis. The molecular mechanisms through which the niche controls the HSC cell cycle to establish quiescence are beginning to be elucidated. For example, it has been shown that the interaction of the Tie2 receptor tyrosine kinase with its ligand Angiopoietin-1 leads to tight adhesion of HSCs to stromal cells, and maintenance of their long-term repopulating activity (Arai et al. 2004). In spite of their generally quiescent state, the normal homeostatic balance in the hematopoietic system requires HSCs to be able to exit the niche and then achieve several transits through vascular endothelium to be able to migrate through the blood, enter different organs and then return back to the bone marrow. These processes of migration and specific homing need to be amplified during stress-induced recruitment of leukocytes from the bone marrow reservoir and during stem cell mobilization as part of defense and repair. Both HSC mobilization (reviewed by Pelus and Fukuda 2008) and homing (reviewed by Lapidot et al. 2005; Chute 2006) are also crucially important in the context of clinical stem cell transplantation.

HSCs induced to exit the bone marrow and mobilize to the peripheral blood following treatment with granulocyte-colony stimulating factor (G-CSF) have become the most widely used source of HSCs for engraftment and show significant superiority to cells obtained directly from the bone marrow. In addition to G-CSF, the growth factor SCF, adhesion molecules such as VLA-4 and P- and E-selectins, chemokines, proteolytic enzymes such as elastase and cathepsin G, and various matrix metalloproteinases (MMPs) have all been shown to have a role in stem cell mobilization. The chemokine stromal-derived factor 1 (SDF-1 or CXCL12) and its receptor CXCR4 are major players involved in the regulation of HSC mobilization and homing. During steady-state homeostasis, CXCR4 is expressed by HSCs and also by stromal cells, which are the main source for SDF-1 in the bone marrow. Stress-induced modulations in SDF-1 and CXCR4 levels participate in recruitment of immature and maturing leukocytes from the bone marrow reservoir to damaged organs as part of host defense and repair mechanisms. The recent finding that murine HSCs rapidly mobilized by the CXCR2 receptor agonist GROβ show superior repopulation kinetics and more competitive engraftment than the equivalent cells mobilized by G-CSF demonstrates that the chemokine/chemokine receptor axis has potentially superior therapeutic potential compared to the use of G-SCF (Fukuda et al. 2007).

In addition to the complex interplay with niche cells and diffusible mediators, it has emerged recently that there is an element of dynamic regulation via neurotransmitter signaling. Hence, ablation by genetic or chemical means of adrenergic neurotransmission or administration of a β2 adrenergic agonist results, respectively, in decreased or enhanced HSC mobilization indicating the involvement of norepinephrine signaling in the process (Katayama et al. 2006; Spiegel et al. 2008).

HSC homing involves rolling and firm adhesion to endothelial cells in small marrow sinusoids under blood flow, followed by trans-endothelial migration across the physical endothelium/extracellular matrix barrier, ultimately leading to access and anchorage to their specialized niches. Like mobilization, this coordinated, multistep process also involves signaling by SDF-1 and SCF, and activation of LFA-1, VLA-4/5 and CD44 and a role for MMPs.

Although HSCs and their niche clearly have to persist throughout life, a number of studies have shown that there are age-related changes in HSCs that have functional consequences for the hematopoietic system and are likely the result of a combination of cell-intrinsic and microenvironmental influences (for a review see Dykstra and de Haan 2008). Studies of X-chromosome inactivation in elderly females have suggested that the pool of HSCs normally diminishes with age, resulting in oligoclonal or even monoclonal hematopoiesis; however, by analyzing the pattern of allele-specific gene expression, Swierczek et al. (2008) have provided convincing evidence against this hypothesis and suggest that clonal hematopoiesis is not a normal consequence of aging. Nevertheless, consideration of HSC differentiation potential suggests that a degree of selection can operate. HSCs isolated from young and aged donors have been reported to differ in functional capacity, the complement of proteins on the cell surface, transcriptional activity, and genome integrity (reviewed by Woolthuis et al. 2011). In the mouse, several hallmark age-dependent changes in the HSC compartment have been identified, including an increase in HSC numbers and a decrease in homing efficiency. Increased proliferation and decreased function with age can be correlated with dramatic alterations in gene expression; one analysis of HSCs from mice aged 2–21 months identified approximately 1,500 genes that were age-induced and 1,600 that were age-repressed (Chambers and Goodell 2007). Genes associated with the stress response, inflammation, and protein aggregation dominated the up-regulated expression profile, while the down-regulated profile was marked by genes involved in the preservation of genomic integrity and chromatin remodeling. One gene in particular that has attracted attention in this context, and may have implications for treatment strategies, is the cyclin-dependent kinase inhibitor p16INK4a, the level of which accumulates and modulates specific age-associated HSC functions (Janzen et al. 2006). Notably, in the absence of p16INK4a, HSC repopulating defects and apoptosis were mitigated, improving the stress tolerance of cells and the survival of animals in successive transplants, suggesting that therapeutic inhibition of genes such as p16INK4a may ameliorate the physiological impact of ageing on stem cells. The differences in ‘aged’ behavior of HSCs were later explained by an accumulation of myeloid-biased HSCs with age in both mice (Challen et al. 2010) and humans (Pang et al. 2011) at the expense of lymphoid-biased cells (Cho et al. 2008). The fact that myeloid-biased

HSCs from young and aged sources behave similarly in all aspects tested might suggest that aging does not change individual HSCs.

There has been a growing appreciation over the last 15 years of the role that reactive oxygen species (ROS) play in a variety of cellular processes. ROS are formed by the partial reduction of oxygen and include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-) (Turrens 2003). ROS have been shown to regulate cell cycle progression, cell motility and growth factor signaling in a variety of normal cell types (Valko et al. 2007). ROS production and consequent oxidative stress has been linked to aging and degenerative disease (Sardina et al. 2012), although beneficial effects of moderate levels of ROS have been noted (Goldstone et al. 1996; Tatla et al. 1999). The importance of ROS in HSCs was made evident from studies on mouse models in which genes involved in the regulation of ROS levels were genetically reduced. For example, reduction of FOXO transcription factor function leads to loss of HSC quiescence and self-renewal capacity (Jang and Sharkis 2007). In the absence of external stimuli, FOXO proteins normally reside in the nucleus in an active state, promoting cell cycle arrest, resistance to stress, apoptosis and ROS detoxification (Coffer and Burgering 2007). Although it is evident that ROS levels are crucial to the function of HSCs, the precise mechanisms affected are not clear. There are actually distinct HSC niches in the bone marrow depending on oxygen availability and the consequent levels of ROS. Hence, ROS^{low} and ROS^{high} HSCs exhibit the same surface phenotype, but differ in that the population with lower ROS levels displays higher self-renewal (Jang and Sharkis 2007). The association between the oxidative state and HSC self-renewal capacity has led to interest in the manipulation of ROS levels as a way to enhance BMT and to delay the aging of HSCs.

10.4 Differentiation Capacity and Their Precursors

What happens downstream of the HSC in the hematopoietic hierarchy is important in a therapeutic context when considering the specific requirements for progenitors and differentiated progeny to regenerate the normal homeostatic state. Work largely emanating from the laboratory of Irving Weissman has defined committed progenitors in the mouse that are immediately downstream of the most mature component of the LSK compartment. These cells mark the first distinction between the lymphoid and myeloid lineages. The existence of a common lymphoid progenitor (CLP) that can only give rise to T cells, B cells, and NK cells was first reported by Kondo et al. (1997), who described a bone marrow Lin^- IL-$7R^+$ Thy-1^- Sca-1^{lo} c-Kit^{lo} population with these characteristics. A complementary clonogenic common myeloid progenitor (CMP) that gives rise to all myeloid lineages was similarly defined by Akashi et al. (2000) who also demonstrated that this cell can give rise to either megakaryocyte/erythrocyte progenitors (MEPs) or granulocyte/macrophage progenitors (GMPs). The resulting model, which proposes that the first lineage commitment step of HSCs results in a strict separation into CLPs and CMPs, has been challenged by the identification of a population of cells with lympho-myeloid

differentiation potential, but that have lost the ability to adopt erythroid and megakaryocyte lineage fates (Adolfsson et al. 2005). Hence, LSK HSCs that co-express high levels of the tyrosine kinase receptor Flt3 were shown to sustain granulocyte, monocyte, and B and T cell potentials, but in contrast to LSK Flt3$^-$ HSCs failed to produce significant erythroid and megakaryocytic progeny. These cells were termed lymphoid-primed multipotent progenitor (LMPP) cells. The equivalent details of the hierarchy downstream of the HSC are yet to be fully elucidated in humans, and it cannot be assumed that these will be comparable between species. Using similar strategies for the identification of down-stream human progenitors, populations corresponding to the mouse LMPP were also defined in humans (MLPs) that sustained both lymphoid and myeloid lineages but excluded megakaryocytic/erythroid potential (Hoebeke et al. 2007; Six et al. 2007; Goardon et al. 2011).

Such details of the pathways of commitment and differentiation of HSCs as described above, and how these may differ during development, are becoming ever more important in attempts to optimize the production of replacement hematopoietic cells from ES cells. As for the definition of the HSC phenotype and functional testing of HSCs in vivo, attempts to elucidate protocols for the induction of hematopoietic differentiation from ES cells have been led by work in the mouse. Although conditions have been worked out to enable the derivation of most mature hematopoietic cell types from both mouse and human ES cells, it is important to be aware of the developmental stage that these cells represent and the extent to which it is possible to generate adult HSCs with repopulation potential (for reviews on the derivation of hematopoietic cells from human ES cells see Bhatia (2007), Tian and Kaufman (2008) and Moreno-Gimeno et al. (2010)). Following on from extensive studies on the differentiation of hematopoietic cells from murine ES cells (reviewed in Olsen et al. 2006), production from human ES cells was first described by Kaufman et al. (2001) who employed co-culture with murine bone marrow stromal or yolk sac endothelial cell lines. Amongst a number of subsequent modifications to this strategy, Vodyanik et al. (2005) were able to obtain large numbers of CD34$^+$ cells at greater than 95% purity using co-culture with the mouse stromal line OP9. Although the latter ES cell-derived CD34$^+$ cells contained ALDH$^+$ Rh-123^{lo} cells and were highly enriched in colony-forming cells, even after in vitro expansion, they displayed a phenotype of primitive hematopoietic progenitors. A potential solution to the problem of the stage of developmental maturity was found in the case of mouse ES cell differentiation in that expression of HoxB4 in primitive progenitors combined with culture on hematopoietic stroma induced a switch to the definitive HSC phenotype capable of engrafting primary and secondary recipients (Kyba et al. 2002).

Encouragingly, since the first successes with human ES cell differentiation into HSC-like cells, conditions have been improved considerably leading ultimately to the in vitro generation of HSCs with repopulation activity. For example, Narayan et al. (2006) were able to engraft sheep using Lin$^-$ CD34$^+$ or CD34$^+$ CD38$^-$ obtained by culturing human ES cells on stromal feeders, their long-term engrafting potential being confirmed by successful transplantation into secondary recipients. Similarly, using co-culture with stromal cells, this time derived from mouse aorta-gonad-mesonephros

(AGM) and fetal liver, Ledran et al. (2008) obtained cells expressing CD34 at day 18–21 of differentiation that were capable of primary and secondary hematopoietic engraftment into immunocompromised mice at substantially higher levels than described previously.

10.5 Potential Applications for Therapies

The utilization of stem cells in the clinic has already met with great success and remains one of the most appealing prospects in regenerative medicine today. The therapeutic use of HSCs pioneered in the 1950s through the development of BMT, initially used matched siblings as donors but has subsequently come to involve the use of partially matched or mismatched donors that although deemed necessary in most situations can result in problems arising from immunogenic matching, resulting in rejection or graft-versus host disease (reviewed by Copelan 2006). A range of diseases have been successfully treated by BMT, including principally cancers of blood cells, but also other hematological disorders such as myeloproliferation, anemia and genetic defects that cause immunodeficiency. BMT is also an option for treatment of some inherited metabolic disorders that result from an enzyme deficiency affecting cells in addition to, or other than, blood cells, but which can be ameliorated through the production of the deficient protein from engrafted donor blood cells. The applications of BMT will be considered in much more detail in Chap. 26, and here the discussion will focus on factors that might improve the prospects for the therapeutic application of HSCs.

Possible improvements in HSC therapies can essentially be broken down into those that increase the availability of suitable, preferably autologous cells in large numbers, and those that maximize the efficiency of engraftment of the transplanted cells. The latter prospect relates to understanding of the mechanisms of homing and the factors that control niche occupancy as discussed above, and it is likely that this knowledge will have a significant impact in the years to come. To date, means to improve the availability of HSCs for BMT have received far more attention. Roughly 30% of patients requiring BMT have a matched sibling, while another 50% potentially have a good match to an individual amongst the nine million registered donors worldwide, although less than half of these will actually receive a donation. Although cord blood is a viable alternative source of HSCs it is not ideal because it only contains a limited number of HSCs, so that ex vivo expansion is almost certainly necessary. Ex vivo expansion of HSCs in combinations of cytokines and other soluble factors, designed to mimic the signals provided within the niche, has met with mixed success, although more recently quite significant degrees of amplification in the numbers of cells retaining engraftment potential have been achieved. For example, using a combination of SCF, Flt3 ligand (FL), thrombopoietin (Tpo) and IL6, two independent groups achieved significant expansion of $CD34^+$ cord blood cells that retained the capacity to repopulate NOD/SCID mice (Kusadasi et al. 2000; Ueda et al. 2000). Direct manipulation of molecular mechanisms that are linked to proliferation and self-renewal is

another potential way to expand stem cells and ectopic over-expression of the transcriptional regulator HoxB4 has proved to be effective at inducing rapid, extensive, and highly polyclonal expansions of murine HSCs that retained full lymphomyeloid repopulating potential and enhanced in vivo regenerative potential (Antonchuk et al. 2002). Other approaches that have been investigated include the use of fibroblast growth factors (FGFs), in particular FGF 1 and 2, which can maintain long-term repopulating activity of mouse bone marrow HSCs in vitro (de Haan et al. 2003; Yeoh et al. 2006), while the Notch ligand Delta 1 has a moderate effect in enhancing the expansion of SRUs in cultures of cord blood $CD133^+$ cells employing the SCF, FL, Tpo, IL-6 cocktail of factors described above (Suzuki et al. 2006). Perhaps the greatest success has come from the laboratory of Harvey Lodish who identified angiopoietin-like 2 and angiopoietin-like 3 proteins as factors produced by HSC-supportive mouse fetal liver $CD3^+$ cells (Zhang et al. 2006). These produced a roughly 30-fold expansion of long-term HSCs in culture, which has subsequently been applied to human cord blood cells by developing a serum-free culture containing SCF, TPO, FGF-1, angiopoietin-like 5, and IGFBP2 (Zhang et al. 2008).

Many believe that the solution to producing more cells for transplantation lies in the derivation of HSCs from alternative sources, and a number of options have been considered in order to achieve this goal. The prospect of in vitro production of HSCs as a futuristic potential supply for BMT derived from ES cells is an exciting opportunity for regenerative medicine as they represent a theoretically unlimited source of HSCs. Nevertheless as for cord blood-derived HSCs there are at present significant limitations to the number of appropriate cells that may be obtained.

As discussed already, ES cells can be differentiated into HSCs with repopulating capability and it may soon be possible to produce these in quantities that are sufficient for clinical application. However, the use of ES cell-derived HSCs is ultimately limited because they are unlikely to be perfectly matched to the donor and ethical consequences of the generation of human embryos for therapeutic applications must be considered. Alternatively, what if ES cells could be made that match every individual so that truly personalized stem cell transplantations would become a reality? Efforts have been made to do just this with ES-like cells being generated through processes such as nuclear transfer, involving either the fusion of ES cells with somatic cell or the transfer of somatic nuclear contents into an oocyte (Wilmut et al. 1997).

The real breakthrough came in 2006 when the Japanese researcher Shinya Yamanaka showed that it is possible to convert normal differentiated adult cells, firstly from the mouse (Takahashi and Yamanaka 2006; Okita et al. 2007) and then from humans (Takahashi et al. 2007), to become like ES cells by forced expression of specific pluripotent genes; namely OCT4, SOX2, KLF4 and c-MYC. These cells, which are usually referred to as induced pluripotent stem (iPS) cells, have the additional advantage that their generation does not involve the use of an embryo bypassing many ethical concerns. The subsequent demonstration that iPS cells can be, like ES cells, differentiated into HSCs (Hanna et al. 2007; Schenke-Layland et al. 2008; Niwa et al. 2009) means that they offer the real prospect of limitless autologous HSCs for all. Of course there are many details yet to be ironed out, but the progress

in this area of stem cell science is nothing if not meteoric (see Hochedlinger and Plath (2009) and Robinton and Daley (2012) for reviews). Interestingly, a recent study has shown that immature hematopoietic cells derived from human iPS cells are more permissive to engraft the bone marrow of xenotransplantation recipients compared to phenotypically identical cells obtained from ES cells, although these HSCs failed to demonstrate multilineage differentiation unless they were removed from the animal, a phenomenon that could be attributed to their inability to down regulate key micro RNAs involved in hematopoiesis (Risueño et al. 2012).

The considerable recent effort in reprogramming cell phenotype towards a pluripotent state has also led to renewed interest in trans-differentiation directly from one cell type to another, without progressing through an iPS cell stage. This has been achieved, with varying degrees of success, for a number of cell types, including neural cells, cardiomyocytes and hepatocytes. One potentially exciting advantage of a trans-differentiation approach is that it may be possible more easily to produce mature cells with an adult rather than embryonic or fetal phenotype. To date, this approach has achieved only limited success with respect to hematopoietic cells, although the laboratory of Mickie Bhatia has been able to demonstrate direct conversion of human fibroblasts to multilineage blood progenitors through ectopic expression of Oct4 (Szabo et al. 2010).

The ability to produce iPS cells, and consequently patient-specific HSCs, also offers the exciting prospect that inherited blood-related disorders might be corrected. Proof-of-principle for this concept was first provided by the laboratory of Rudi Jaenisch who created a humanized sickle cell anemia mouse model, which was then rescued after transplantation with HSCs obtained in vitro from autologous iPS cells in which the mutant hemoglobin allele had been reverted to the normal sequence by gene-specific targeting (Hanna et al. 2007). A second important proof-of-principle, this time with human cells, has been the demonstration that somatic cells from Fanconi anemia patients can be used to generate iPS cells in which the Fanconi-anemia defect could be corrected by over expression of the normal version of the affected protein and then used to give rise to hematopoietic progenitors of the myeloid and erythroid lineages that are disease-free (Raya et al. 2009).

Although these exciting advances in iPS cell technology generate the prospect of patient-specific stem cell therapies, the transition from the bench to the patient bedside is still some distance into the future. Numerous obstacles must be overcome before these therapies are put into routine practice. Firstly, the original methods of iPS cell generation utilized retroviruses as the vectors to infect cells to initiate the expression of the pluripotent genes. This process would be entirely unacceptable in the clinic since retroviruses are known cancer-causing agents (Okita et al. 2007). New methods of generation of iPS cells have evolved with the means to remove the oncogenes after the induction of pluripotency reducing the risk of tumorigenesis (Yu et al. 2009).

Although it is some distance into the future before these techniques are put into clinical practice, the generation of HSCs from iPS cells from patients can be used in the present for phenotypic based drug screens in complex diseases for which the under-lying genetic mechanism is unknown.

10.6 Conclusions and Future Development in Research

Research into the nature and application of HSCs has come a long way since the earliest forays into transplantation of bone marrow into patients. Apart from the highly detailed understanding that we now have of the molecular and cellular characteristics of HSCs and ways in which they can be manipulated and used for clinical benefit, research in this area has provided the guiding light for the whole field of stem cell biology. The means of identifying and purifying HSCs and the sophisticated in vitro and in vivo tests that have been developed to assay their potential have been adopted and modified for the investigation of the now burgeoning array of stem cells that play roles in both development and the maintenance of adult tissues. The study of HSCs illustrates so well how investigations in animal models, in particular the mouse, can inform studies in man and how they can provide important pre-clinical information on the potency and behaviour of stem cells following ex vivo expansion or on ways in which to improve the efficiency of engraftment once cells are introduced into the recipient.

The improvements in the ability of clinicians to treat patients more effectively with transplantations as a result of the increasing knowledge of HSCs are likely to be given an even greater boost as a result of the astonishing developments in the ability to generate pluripotent stem cells and to then use these to produce HSCs with long-term engraftment potential. Apart from the chance to treat more people successfully, there has now opened up the real prospect that individuals born with genetic defects that affect blood cell production or function, as well as some other inherited disorders such as those affecting aspects of metabolism, can expect to have their deficiencies corrected by gene targeting in iPS cells generated from nothing more than a few skins cells.

For sure there are many hurdles yet to be overcome, but the future looks very exciting.

References

Adams GB, Scadden DT (2008) A niche opportunity for stem cell therapeutics. Gene Ther 15:96–99

Adolfsson J, Borge OJ, Bryder D et al (2001) Upregulation of Flt3 expression within the bone marrow Lin(−)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity 15:659–669

Adolfsson J, Månsson R, Buza-Vidas N et al (2005) Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell 121:295–306

Akashi K, Traver D, Miyamoto T et al (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404:193–197

Almeida-Porada G, Porada C, Gupta N et al (2007) The human-sheep chimeras as a model for human stem cell mobilization and evaluation of hematopoietic grafts' potential. Exp Hematol 35:1594–1600

Ando K (2002) Human CD34- hematopoietic stem cells: basic features and clinical relevance. Int J Hematol 75:370–375
in ‘t Anker PS, Noort WA, Kruisselbrink AB et al (2003) Nonexpanded primary lung and bone marrow-derived mesenchymal cells promote the engraftment of umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. Exp Hematol 31:881–889
Antonchuk J, Sauvageau G, Humphries RK (2002) HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell 109:39–45
Arai F, Hirao A, Ohmura M et al (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118:149–161
Balazs AB, Fabian AJ, Esmon CT et al (2006) Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow. Blood 107:2317–2321
Baum CM, Weissman IL, Tsukamoto AS et al (1992) Isolation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci USA 89:2804–2808
Bensidhoum M, Chapel A, Francois S et al (2004) Homing of in vitro expanded Stro-1– or Stro-1+ mesenchymal stem cells into the NOD/SCID mouse and their role in supporting human CD34 cell engraftment. Blood 103:3313–3319
Bhatia M (2007) Hematopoietic development from human embryonic stem cells. Hematol Am Soc Hematol Educ Progr 2007:11–16
Bhatia M, Wang JC, Kapp U et al (1997) Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci USA 94:5320–5325
Bhatia M, Bonnet D, Murdoch B et al (1998) A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat Med 4:1038–1045
Bonnet D, Bhatia M, Wang JC et al (1999) Cytokine treatment or accessory cells are required to initiate engraftment of purified primitive hematopoietic cells transplanted at limiting doses into NOD/SCID mice. Bone Marrow Transplant 23:203–209
Bradley TR, Metcalf D (1966) The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci 44:287–299
Challen GA, Boles NC, Lin KK et al (2009) Mouse hematopoietic stem cell identification and analysis. Cytometry A 75:14–24
Challen GA, Boles NC, Chambers SM et al (2010) Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. Cell Stem Cell 6:265–278
Chambers SM, Goodell MA (2007) Hematopoietic stem cell aging: wrinkles in stem cell potential. Stem Cell Rev 3:201–211
Chen BP, Galy A, Kyoizumi S et al (1994) Engraftment of human hematopoietic precursor cells with secondary transfer potential in SCID-hu mice. Blood 84:2497–2505
Chen CZ, Li L, Li M et al (2003) The endoglin(positive) sca-1(positive) rhodamine(low) phenotype defines a near-homogeneous population of long-term repopulating hematopoietic stem cells. Immunity 19:525–533
Cho RH, Sieburg HB, Muller-Sieburg CE (2008) A new mechanism for the aging of hematopoietic stem cells: aging changes the clonal composition of the stem cell compartment but not individual stem cells. Blood 111:5553–5561
Christensen JL, Weissman IL (2001) Flk-2 is a marker in hematopoietic stem cell differentiation: a simple model to isolate long-term stem cells. Proc Natl Acad Sci USA 98:14541–14546
Chute JP (2006) Stem cell homing. Curr Opin Hematol 13:399–406
Coffer PJ, Burgering BM (2007) Stressed marrow: FoxOs stem tumour growth. Nat Cell Biol 9:251–253
Copelan EA (2006) Hematopoietic stem-cell transplantation. N Engl J Med 354:1813–1826
Craig W, Kay R, Cutler RL et al (1993) Expression of Thy-1 on human hematopoietic progenitor cells. J Exp Med 177:1331–1342
Cumano A, Godin I (2007) Ontogeny of the hematopoietic system. Annu Rev Immunol 25:745–785
de Haan G, Weersing E, Dontje B et al (2003) In vitro generation of long-term repopulating hematopoietic stem cells by fibroblast growth factor-1. Dev Cell 4:241–251

Dexter TM, Allen TD, Lajtha LG (1977) Conditions controlling the proliferation of hematopoietic stem cells in vitro. J Cell Physiol 91:335–344
Dick JE, Magli MC, Huszar D et al (1985) Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/Wv mice. Cell 42:71–79
Doulatov S, Notta F, Laurenti E et al (2012) Hematopoiesis: a human perspective. Cell Stem Cell 10:120–136
Dykstra B, de Haan G (2008) Hematopoietic stem cell aging and self-renewal. Cell Tissue Res 331:91–101
Dzierzak E, Speck NA (2008) Of lineage and legacy: the development of mammalian hematopoietic stem cells. Nat Immunol 9:129–136
Dzierzak E, Medvinsky A, de Bruijn M (1998) Qualitative and quantitative aspects of haematopoietic cell development in the mammalian embryo. Immunol Today 19:228–236
Ema H, Takano H, Sudo K et al (2000) In vitro self-renewal division of hematopoietic stem cells. J Exp Med 192:1281–1288
Engelhardt M, Lübbert M, Guo Y (2002) CD34(+) or CD34(−): which is the more primitive? Leukemia 16:1603–1608
Fraser CC, Chen BP, Webb S et al (1995) Circulation of human hematopoietic cells in severe combined immunodeficient mice after Cl2MDP-liposome-mediated macrophage depletion. Blood 86:183–192
Fukuda S, Bian H, King AG et al (2007) The chemokine GRObeta mobilizes early hematopoietic stem cells characterized by enhanced homing and engraftment. Blood 110:860–869
Gallacher L, Murdoch B, Wu DM et al (2000) Isolation and characterization of human CD34(−) Lin(−) and CD34(+)Lin(−) hematopoietic stem cells using cell surface markers AC133 and CD7. Blood 95:2813–2820
Goardon N, Marchi E, Atzberger A et al (2011) Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. Cancer Cell 19:138–152
Goldman JP, Blundell MP, Lopes L et al (1998) Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. Br J Haematol 103:335–342
Goldstone SD, Milligan AD, Hunt NH (1996) Oxidative signalling and gene expression during lymphocyte activation. Biochim Biophys Acta 1314:175–182
Goodell MA, Brose K, Paradis G et al (1996) Isolation and functional properties of murine hemopoietic stem cells that are replicating in vivo. J Exp Med 183:1797–1806
Goodell MA, Rosenzweig M, Kim H et al (1997) Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 3:1337–1345
Greiner DL, Hesselton RA, Shultz LD (1998) SCID mouse models of human stem cell engraftment. Stem Cells 16:166–177
Guo Y, Lübbert M, Engelhardt M (2003) CD34- hematopoietic stem cells: current concepts and controversies. Stem Cells 21:15–20
Hanna J, Wernig M, Markoulaki S et al (2007) Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science 318:1920–1923
Hao QL, Shah AJ, Thiemann FT et al (1995) A functional comparison of CD34+ CD38− cells in cord blood and bone marrow. Blood 86:3745–3753
Harrison DE, Jordan CT, Zhong RK et al (1993) Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple bionomial, correlation and covariance calculations. Exp Hematol 21:206–219
Hess DA, Wirthlin L, Craft TP et al (2006) Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. Blood 107:2162–2169
Hochedlinger K, Plath K (2009) Epigenetic reprogramming and induced pluripotency. Development 136:509–523
Hoebeke I, De Smedt M, Stolz F et al (2007) T-, B- and NK-lymphoid, but not myeloid cells arise from human CD34(+)CD38(−)CD7(+) common lymphoid progenitors expressing lymphoid-specific genes. Leukemia 21:311–319

Ishii M, Matsuoka Y, Sasaki Y et al (2011) Development of a high-resolution purification method for precise functional characterization of primitive human cord blood-derived CD34-negative SCID-repopulating cells. Exp Hematol 39:203–213
Ishikawa F, Yasukawa M, Lyons B et al (2005) Development of functional human blood and immune systems in NOD/SCID/IL2 receptor gamma chain(null) mice. Blood 106:1565–1573
Ito M, Hiramatsu H, Kobayashi K et al (2002) NOD/SCIDgamma(c) (null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 100:3175–3182
Ito M, Kobayashi K, Nakahata T (2008) NOD/Shi-scid IL2rgamma(null) (NOG) mice are more appropriate for humanized mouse models. Curr Top Microbiol Immunol 324:53–76
Jang YY, Sharkis SJ (2007) A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood 110:3056–3063
Janzen V, Forkert R, Fleming HE et al (2006) Stem cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 443:42142–42146
Kamel-Reid S, Dick JE (1988) Engraftment of immune-deficient mice with human hematopoietic stem cells. Science 242:1706–1709
Kastan MB, Schlaffer E, Russo JE et al (1990) Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. Blood 75:1947–1950
Katayama Y, Battista M, Kao WM et al (2006) Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell 124:407–421
Kaufman DS, Hanson ET, Lewis RL et al (2001) Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci USA 98:10716–10721
Kent DG, Copley MR, Benz C et al (2009) Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. Blood 113:6342–63450
Kiel MJ, Morrison SJ (2008) Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol 8:290–301
Kiel MJ, Yilmaz OH, Iwashita T et al (2005) SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121:1109–1121
Kimura T, Asada R, Wang J et al (2007) Identification of long-term repopulating potential of human cord blood-derived CD34-flt3- severe combined immunodeficiency-repopulating cells by intra-bone marrow injection. Stem Cells 25:1348–1355
Kollet O, Peled A, Byk TB et al (2000) Beta2 microglobulin-deficient (B2m(null)) NOD/SCID mice are excellent recipients for studying human stem cell function. Blood 95:3102–3105
Kondo M, Weissman IL, Akashi K (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91:661–672
Kusadasi N, van Soest PL, Mayen AE et al (2000) Successful short-term ex vivo expansion of NOD/SCID repopulating ability and CAFC week 6 from umbilical cord blood. Leukemia 14:1944–1953
Kushida T, Inaba M, Hisha H et al (2001) Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 97:3292–3299
Kyba M, Perlingeiro RC, Daley GQ (2002) HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell 109:29–37
Lai AY, Kondo M (2006) Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. J Exp Med 203:1867–1873
Lapidot T, Pflumio F, Doedens M et al (1992) Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. Science 255:1137–1141
Lapidot T, Dar A, Kollet O (2005) How do stem cells find their way home? Blood 106:1901–1910
Larochelle A, Savora M, Wiggins M et al (2011) Human and rhesus macaque hematopoietic stem cells cannot be purified based only on SLAM family markers. Blood 117:1550–1554
Ledran MH, Krassowska A, Armstrong L et al (2008) Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches. Cell Stem Cell 3:85–98
Lemieux ME, Rebel VI, Lansdorp PM et al (1995) Characterization and purification of a primitive hematopoietic cell type in adult mouse marrow capable of lymphomyeloid differentiation in long-term marrow “switch” cultures. Blood 86:1339–1347

Lemischka IR, Raulet DH, Mulligan RC (1986) Developmental potential and dynamic behavior of hematopoietic stem cells. Cell 45:917–927
Li Z, Li L (2006) Understanding hematopoietic stem-cell microenvironments. Trends Biochem Sci 31:589–595
Lo Celso C, Fleming HE, Wu JW et al (2009) Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature 457:92–96
Majeti R, Park CY, Weissman IL (2007) Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell 1:635–645
Mayani H, Dragowska W, Lansdorp PM (1993) Cytokine-induced selective expansion and maturation of erythroid versus myeloid progenitors from purified cord blood precursor cells. Blood 81:3252–3258
Mazurier F, Fontanellas A, Salesse S et al (1999) A novel immunodeficient mouse model RAG2 x common cytokine receptor gamma chain double mutants requiring exogenous cytokine administration for human hematopoietic stem cell engraftment. J Interferon Cytokine Res 19:533–541
McCune JM, Namikawa R, Kaneshima H et al (1988) The SCID-hu mouse: murine model for the analysis of human hematoplymphoid differentiation and function. Science 241:1632–1639
McKenzie JL, Gan OI, Doedens M et al (2005) Human short-term repopulating stem cells are efficiently detected following intrafemoral transplantation into NOD/SCID recipients depleted of CD122+ cells. Blood 106:1259–1261
McKenzie JL, Gan OI, Doedens M et al (2006) Individual stem cells with highly variable proliferation and self-renewal properties comprise the human hematopoietic stem cell compartment. Nat Immunol 7:1225–1233
McKenzie JL, Takenaka K, Gan OI et al (2007) Low rhodamine 123 retention identifies long-term human hematopoietic stem cells within the Lin-CD34+CD38− population. Blood 109:543–545
Mercier FE, Ragu C, Scadden DT (2011) The bone marrow at the crossroads of blood and immunity. Nat Rev Immunol 12:49–60
Mikkola HK, Orkin SH (2006) The journey of developing hematopoietic stem cells. Development 133:3733–3744
Moore MA, Williams N, Metcalf D (1973) In vitro colony formation by normal and leukemic human hematopoitic cells: characterization of the colony-forming cells. J Natl Cancer Inst 50:603–623
Moreno-Gimeno I, Ledran MH, Lako M (2010) Hematopoietic differentiation from human ESCs as a model for developmental studies and future clinical translations. Invited review following the FEBS anniversary prize received on 5 July 2009 at the 34th FEBS congress in Prague. FEBS J 277:5014–5025
Namikawa R, Weilbaecher KN, Kaneshima H et al (1990) Long-term human hematopoiesis in the SCID-hu mouse. J Exp Med 172:1055–1063
Narayan AD, Chase JL, Lewis RL et al (2006) Human embryonic stem cell-derived hematopoietic cells are capable of engrafting primary as well as secondary fetal sheep recipients. Blood 107:2180–2183
Niwa A, Umeda K, Chang H et al (2009) Orderly hematopoietic development of induced pluripotent stem cells via Flk-1(+) hemangiogenic progenitors. J Cell Physiol 221:367–377
Nolta JA, Hanley MB, Kohn DB (1994) Sustained human hematopoiesis in immunodeficient mice by cotransplantation of marrow stroma expressing human interleukin-3: analysis of gene transduction of long-lived progenitors. Blood 83:3041–3051
Noort WA, Kruisselbrink AB, in ‘t Anker PS et al (2002) Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. Exp Hematol 30:870–878
Notta F, Doulatov S, Laurenti E et al (2011) Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science 333:218–221
Okada S, Nakauchi H, Nagayoshi K et al (1992) In vivo and in vitro stem cell function of c-kit and Sca-1-positive murine hematopoietic cells. Blood 80:3044–3050

Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448:313–317
Olsen AL, Stachura DL, Weiss MJ (2006) Designer blood: creating hematopoietic lineages from embryonic stem cells. Blood 107:1265–1275
Osawa M, Hanada K, Hamada H et al (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273:242–245
Pang WW, Price EA, Sahoo D et al (2011) Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. Proc Natl Acad Sci USA 108:20012–20017
Pearson T, Greiner DL, Shultz LD (2008) Humanized SCID mouse models for biomedical research. Curr Top Microbiol Immunol 324:25–51
Pelus LM, Fukuda S (2008) Chemokine mobilized adult stem cells: defining a better hematopoietic graft. Leukemia 22:466–473
Ploemacher RE, van der Sluijs JP, Voerman JS et al (1989) An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood 74:2755–2763
Ploemacher RE, van der Sluijs JP, van Beurden CA et al (1991) Use of limiting-dilution type long-term marrow cultures in frequency analysis of marrow-repopulation and spleen colony-forming hematopoietic stem cells in the mouse. Blood 78:2527–2533
Pronk CJ, Rossi DJ, Mansson R et al (2007) Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor hierarchy. Cell Stem Cell 1:428–442
Purton LE, Scadden DT (2007) Limiting factors in murine hematopoietic stem cell assays. Cell Stem Cell 1:263–270
Raaijmakers MH, Scadden DT (2008) Evolving concepts on the microenvironmental niche for hematopoietic stem cells. Curr Opin Hematol 15:301–306
Raya A, Rodríguez-Pizà I, Guenechea G et al (2009) Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature 460:53–59
Risueño RM, Sachlos E, Lee JH et al (2012) Inability of human induced pluripotent stem cell-hematopoietic derivatives to downregulate micro RNAs in vivo reveals a block in xenograft hematopoietic regeneration. Stem Cells 30:131–139
Robinton DA, Daley GQ (2012) The promise of induced pluripotent stem cells in research and therapy. Nature 481:295–305
Rongvaux A, Willinger T, Takizawa H et al (2011) Human thrombopoietin knockin mice efficiently support human hematopoiesis in vivo. Proc Natl Acad Sci USA 108:2378–2383
Sardina JL, López-Ruano G, Sánchez-Sánchez B et al (2012) Reactive oxygen species: are they important for hematopoiesis? Crit Rev Oncol Hematol 81:257–274
Schenke-Layland K, Rhodes KE, Angelis E et al (2008) Reprogrammed mouse fibroblasts differentiate into cells of the cardiovascular and hematopoietic lineages. Stem Cells 26:1537–1546
Schmitt TM, Zúñiga-Pflücker JC (2002) Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity 17:749–756
Shultz LD, Schweitzer PA, Christianson SW et al (1995) Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. J Immunol 154:180–191
Sintes J, Romero X, Marin P et al (2008) Differential expression of CD150 (SLAM) family receptors by human hematopoietic stem and progenitor cells. Exp Hematol 36:1199–1204
Sitnicka E, Buza-Vidas N, Larsson S et al (2003) Human CD34+ hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express flt3: distinct flt3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cells. Blood 102:881–886
Six EM, Bonhomme D, Monteiro M et al (2007) A human postnatal lymphoid progenitor capable of circulating and seeding the thymus. J Exp Med 204:3085–3093
Spangrude GJ, Johnson GR (1990) Resting and activated subsets of mouse multipotent hematopoietic stem cells. Proc Natl Acad Sci USA 87:7433–7437
Spangrude GJ, Heimfeld S, Weissman IL (1988) Purification and characterization of mouse hematopoietic stem cells. Science 241:58–62
Spiegel A, Kalinkovich A, Shivtiel S et al (2008) Stem cell regulation via dynamic interactions of the nervous and immune systems with the microenvironment. Cell Stem Cell 3:484–492

Srour EF, Zanjani ED, Cornetta K et al (1993) Persistence of human multilineage, self-renewing lymphohematopoietic stem cells in chimeric sheep. Blood 82:3333–3342
Storms RW, Trujillo AP, Springer JB et al (1999) Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc Natl Acad Sci USA 96:9118–9123
Sutherland HJ, Eaves CJ, Eaves AC et al (1989) Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood 74:1563–1570
Suzuki T, Yokoyama Y, Kumano K et al (2006) Highly efficient ex vivo expansion of human hematopoietic stem cells using Delta1-Fc chimeric protein. Stem Cells 24:245624–245665
Swierczek SI, Agarwal N, Nussenzveig RH et al (2008) Hematopoiesis is not clonal in healthy elderly women. Blood 112:3186–3193
Szabo E, Rampalli S, Risueño RM et al (2010) Direct conversion of human fibroblasts to multilineage blood progenitors. Nature 468:521–526
Szilvassy SJ, Humphries RK, Lansdorp PM et al (1990) Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. Proc Natl Acad Sci USA 87:8736–8740
Taichman RS (2005) Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. Blood 105:2631–2639
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
Takahashi K, Tanabe K, Ohnuki M et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
Takano H, Ema H, Sudo K et al (2004) Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med 199:295–302
Tatla S, Woodhead F et al (1999) The role of reactive oxygen species in triggering proliferation and IL-2 secretion in T cells. Free Radic Biol Med 26:14–24
Thomas ED, Lochte HL Jr, Lu WC et al (1957) Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. N Engl J Med 257:491–496
Tian X, Kaufman DS (2008) Differentiation of embryonic stem cells towards hematopoietic cells: progress and pitfalls. Curr Opin Hematol 15:312–318
Till JE, McCulloch EA (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14:213–222
Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552:335–344
Ueda T, Tsuji K, Yoshino H et al (2000) Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. J Clin Invest 105:1013–1021
Valko M, Leibfritz D, Moncol J et al (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44–84
van Os R, Kamminga LM, de Haan G (2004) Stem cell assays: something old, something new, something borrowed. Stem Cells 22:118111–118190
van Rijn RS, Simonetti ER, Hagenbeek A et al (2003) A new xenograft model for graft-versus-host disease by intravenous transfer of human peripheral blood mononuclear cells in RAG-/- gammac-/- double-mutant mice. Blood 102:2522–2531
Vodyanik MA, Bork JA, Thomson JA et al (2005) Human embryonic stem cell-derived CD34+ cells: efficient production in coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood 105:617–626
Wang JC, Doedens M, Dick JE (1997) Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating assay. Blood 89:3919–3924
Wang J, Kimura T, Asada R et al (2003) SCID-repopulating cell activity of human cord blood-derived CD34- cells assured by intra-bone marrow injection. Blood 101:2924–2931
Whitlock CA, Witte ON (1982) Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc Natl Acad Sci USA 79:3608–3612

Willinger T, Rongvaux A, Stowig T et al (2011) Improving human hemato-lymphoid-system mice by cytokine knock-in gene replacement. Trends Immunol 32:321–327
Wilmut I, Schnieke AF, McWhir J et al (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813
Wilson A, Trumpp A (2006) Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol 6:93–106
Woolthuis CM, de Haan G, Huls G (2011) Aging of hematopoietic stem cells: Intrinsic changes or micro-environmental effects? Curr Opin Immunol 23:512–517
Yahata T, Ando K, Sato T et al (2003) A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. Blood 101:2905–2913
Yang L, Bryder D, Adolfsson J et al (2005) Identification of Lin(−)Sca1(+)kit(+)CD34(+)Flt3-short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. Blood 105:2717–2723
Yeoh JS, van Os R, Weersing E et al (2006) Fibroblast growth factor-1 and -2 preserve long-term repopulating ability of hematopoietic stem cells in serum-free cultures. Stem Cells 24:1564–1572
Yin AH, Miraglia S, Zanjani ED et al (1997) AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 90:5002–5012
Yu J, Hu K, Smugga-Otto K et al (2009) Human induced pluripotent stem cells free of vector and transgene sequences. Science 324:797–801
Zanjani ED, Pallavicini MG, Ascensao JL et al (1992) Engraftment and long-term expression of human fetal hematopoietic stem cells in sheep following transplantation in utero. J Clin Invest 89:1178–1188
Zanjani ED, Srour EF, Hoffman R (1995) Retention of long-term repopulating ability of xenogeneic transplanted purified adult human bone marrow hematopoietic stem cells in sheep. J Lab Clin Med 126:24–28
Zanjani ED, Almeida-Porada G, Livingston AG et al (1998) Human bone marrow CD34− cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34+ cells. Exp Hematol 26:353–360
Zhang CC, Kaba M, Ge G et al (2006) Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nat Med 12:240–245
Zhang CC, Kaba M, Iizuka S et al (2008) Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. Blood 111:3415–3423

Chapter 11
Cardiovascular Stem Cells

Christoph Brenner, Robert David, and Wolfgang-Michael Franz

Abstract Ischemic disorders are the main cause of death in the Western world. With more patients surviving their acute myocardial infarction and an aging population, congestive heart failure is the rising health problem. At present, heart transplantation remains the only curative treatment for end stage heart failure. The discrepancy between demand and supply of donor organs does not fill the clinical need. This explains the huge effort made in the field of stem cell research trying to establish alternative resources for tissue replacement. In contrast to adult stem cells mainly acting in a paracrine fashion pluripotent stem cells have the potential to generate transplantable myocardial and vascular tissue.

Due to the low percentage of cardiovascular progenitor cells in pluripotent stem cell cultures, various approaches using exogenous factors aim for their amplification and purification in vitro. However, one future key technology may be genetic forward programming based on profound understanding of differentiation pathways in order to direct stem cell differentiation towards cardiovascular fates. In this regard, subtype specific programming has already been achieved by overexpression of distinct early cardiovascular transcription factors leading to populations of either predominantly early/intermediate type cardiomyocytes or differentiated ventricular myocardial cells, respectively.

In addition, techniques for gentle purification of myocardial and vascular progenitor cells will have to be further refined in order to enable the generation of highly specific, pure and safe cell populations for transplantations and for tissue engineering.

C. Brenner, M.D. • R. David, Ph.D. • W.-M. Franz, M.D. (✉)
Department of Internal Medicine I, University of Munich – Campus Grosshadern,
Marchioninistrasse 15, D-81377 Munich, Germany
e-mail: Wolfgang.Franz@med.uni-muenchen.de

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_11,

11.1 Introduction

Cardiovascular diseases are the leading cause of death in the Western world. For instance, in the year 2009 a 42% mortality was related to chronic ischemic cardiomyopathy, myocardial infarction or progressive congestive heart failure in Germany. And this trend is even likely to climb in the foreseeable future (DESTATIS 2010). The loss of cardiomyocytes irreversibly leads to adverse remodeling of the myocardium and chamber dilatation associated with a decreased ejection fraction and heart insufficiency. And with more patients surviving their acute myocardial infarction and a senescent population, congestive heart failure has already become a major health concern. Today, one of the main obstacles responsible for the high mortality of cardiovascular diseases are the limited therapeutical options available reflected by a 1-year-mortality of 50% for patients suffering from end-stage heart failure (NYHA stage III or IV) (Kessler and Byrne 1999). Furthermore, the lack of donor organs for heart transplantations, which is the only causal therapy available, aggravates this shortage of therapeutical options. And in case of successful transplantation allograft vasculopathy and chronic heart transplant rejection are the causes for a poor clinical outcome (Boyle and Colvin-Adams 2004; Hunt 1998; Neumayer 2005). The direct medical costs for treatment of cardiovascular diseases within the EU are estimated at more than 100 billion Euros per year (Leal et al. 2006).

Thus, scientists are encouraged to seek for new therapeutic options. Currently, the main objective is to find a way increasing the regeneration capacity of a diseased organ instead of "simply" replacing it. Therefore, several types of stem or progenitor cells have been introduced, characterized and tested in animal models. The transplantation of skeletal myoblasts in ischemic cardiomyopathy was a promising approach as these autologous cells are easily accessible and available in a sufficient quantity (Menasche et al. 2003). Unfortunately, this non-cardiac muscular tissue did not electrically couple to the working myocardium (Menasche 2005). After transplantation of fetal cardiomyocytes in a mouse model the formation of intercalated discs and long term survival postengraftment could be documented (Soonpaa et al. 1994) so that this cell type could be regarded as a possible source for cell therapy. Merely the clinical use of these cells is hampered by the lacking availability, at least for ethical reasons. Only the use of autologous bone-marrow-derived stem cells is not impaired by the problems mentioned above. Both the surgical implantation of these cells as well as the mobilization in combination with improved homing or the direct cell-application via heart catheter into the ischemic myocardium appeared to be feasible, save and ethically inoffensive (Engelmann et al. 2009; Orlic et al. 2001; Schachinger et al. 2006; Theiss et al. 2010; Zaruba et al. 2009). Furthermore, encouraging results could be generated in the animal models and the clinical trials performed so far (Schachinger et al. 2006; Strauer et al. 2002; Wollert et al. 2004). However, the underlying mechanisms still remain unclear. At least, it appears to be more than unlikely that bone-marrow-derived stem cells can transdifferentiate into a myocardial cell line even though first observations claimed to demonstrate that (Balsam et al. 2004; Orlic et al. 2001). It rather seems that the beneficial effects

caused by these adult stem cells are based on paracrine influence on the surrounding tissue resulting in neo-angiogenesis, anti-apoptosis and probably stimulated proliferation of resident progenitor cells (Deindl et al. 2006; Murry et al. 2004). In summary, bone-marrow-derived stem cells seem to have a positive effect on the healing process of the damaged myocardium but are not a potential source for cardiovascular tissue engineering as this would require the formation of cardiomyocytes, smooth muscle and endothelial cells.

This is the reason why the best source for the generation of myocardial replacement tissue at present are pluripotent stem cells. These cells, depending on their derivation, have the ability to form all different cell types required to form myocardial (atria, ventricle and stroma) and vascular tissue (endothelial and smooth muscle cells) or specialized cardiac cells (e.g. pacemaker, conduction-system) and can be generated as autologous, i.e. genetically compatible to the recipient, stem cells in a theoretically infinite amount (Maltsev et al. 1993). Since the technique of tissue engineering, i.e. the formation of transplantable three-dimensional constructions from beating cardiomyocytes, e.g. by *biological assembly* (Akins et al. 1999) or the *cell sheet approach* (Okano et al. 1995), is already well established and has been refined over the past 15 years (see Chaps. 20 and 21), it is now time to focus on the identification of the appropriate cell types from the pluripotent stem cell culture. Possible applications among others are infarct repair (Bel et al. 2010; Kraehenbuehl et al. 2011; Singla et al. 2011) or the generation of biological pacemakers (Kleger et al. 2010; Shiba et al. 2009). While the use of autologous and patient-specific inducible pluripotent stem cells (iPS-cells, see Chap. 8) will most likely solve the problem of immunological tolerance of the transplanted tissue there still exist some more hurdles that have to be overcome. The generation of sufficient amounts of transplantable cells is one main goal as cardiomyocytes make up less than 10% of all cells in the murine embryonic stem cell culture (Yuasa et al. 2005) and even less in the human ES cell system (Xu et al. 2008). Furthermore, the identification of the appropriate subtypes within the vast diversity of developing cardiovascular cells and their developmental stages within the pluripotent stem cell culture is crucial to achieve the best functional results after transplantation. And last but not least the isolation methods have to be further improved to guarantee the generation of pure and distinct graft cells for transplantation to minimize the risk for cardiac arrhythmias or even teratoma formation (see Chap. 7) (Liao et al. 2010; Lin et al. 2010).

With the identification of stage- and lineage-specific markers and progressing decipherment of the molecular cardiovascular development various opportunities for meeting the challenges mentioned above will appear.

11.2 Characteristics and Classification

The development of the cardiovascular system, i.e. the first organ system to develop in vertebrate embryos, begins with the gastrulation in the third week of human embryonic development. Due to its considerable size at this point in time the embryo

is not able to nourish itself by diffusion alone any more but needs circulating blood for the supply of the highly proliferative tissues (Eisenberg and Eisenberg 2006). While the heart itself represents the first functioning organ in the mammalian body its development begins at day 19 in the cardiac neural crest and the bilateral cardiogenic zones in the anterior visceral mesoderm with the induction of angioblasts by the endoderm (Buckingham et al. 2005; Sadler 1998). At the beginning of the fourth week of embryonic development forming endothelial cell clusters connect to endocardial tubes, translocate into the thoracic region and form the primitive heart tube with an inner endocardial layer and a surrounding myocard (Gerecht-Nir et al. 2003; Sadler 1998). During weeks 5–7 the four chambered heart develops by folding and septum formation and starts beating after connection to the first vascular loops (Larsen 1998). The myocardial derivation from angioblasts and endothelial cell clusters thereby clearly shows that heart and vessels arise from the same original cardiovascular progenitor cells.

However, regarding the cardiovascular development from a molecular point of view is even more complicated. It is well-known that the common cardiovascular stem cells can be derived in various developmental stages from all available pluripotent cell lines like embryonic (see Chap. 7), induced pluripotent (see Chap. 8), spermatogonial (see Chap. 9) or parthenogenetic stem cells (Yamanaka 2007). These pluripotent stem cells show an in vitro differentiation roughly comparable even though not identical to the embryonic development in vivo. And as these cells in the native state express the pluripotency markers Oct3/4, Nanog, Sox etc. (Srivastava and Ivey 2006; Takahashi and Yamanaka 2006) they are all able to form early cardiac mesoderm which is the prerequisite for the development of cardiovascular organs and the production of cardiovascular stem cells (see Fig. 11.1).

While forming the cardiogenic regions in the lateral-plate mesoderm the cardiovascular precursors downregulate the earliest pan-mesodermal marker Brachyury (Bra) and start expressing the transcription factor mesoderm posterior 1 (MesP1) which is highly specific for all cardiogenic and several vasculogenic regions giving rise to the dorsal aorta, intersomitic and cranial vessels (Kitajima et al. 2000; Saga et al. 1999, 2000). Interestingly, Brachyury directly mediates the expression of MesP1 in the cardiovascular progenitor cells by binding to the MesP1 promotor region (David et al. 2011). This explains why MesP1 can serve as a first target gene

Fig. 11.1 (continued) helix-loop-helix transcription factor *mesoderm posterior 1* (MesP1), which is expressed in early cardiovascular progenitor cells determined to form myocardium or blood vessels. (**d**) Whereas vascular progenitors are further characterized by the expression of Islet1 (Isl1), HOXB5 and the VEGF-receptor 2 (Flk-1), myocardial precursors follow a genetic program determined by the cardiac transcriptional key factor Nkx2.5. The further development of the heart can be distinguished between the first and the second heart field, which are characterized by the expression of Tbx20, Tbx5, Nkx2.5, Hand1 and Isl1, FGF8, Mef2c and Tbx1, respectively. Fully differentiated vascular structures express the structural proteins SM-actin and SM-MHC in the smooth muscle cells and CD31 and VE-Cadherin in endothelial cells. Typical cardiac structural proteins are myosin heavy chain (αMHC), ventricular myosin light chain (MLC2v), Troponin and Connexin40 for electrical coupling

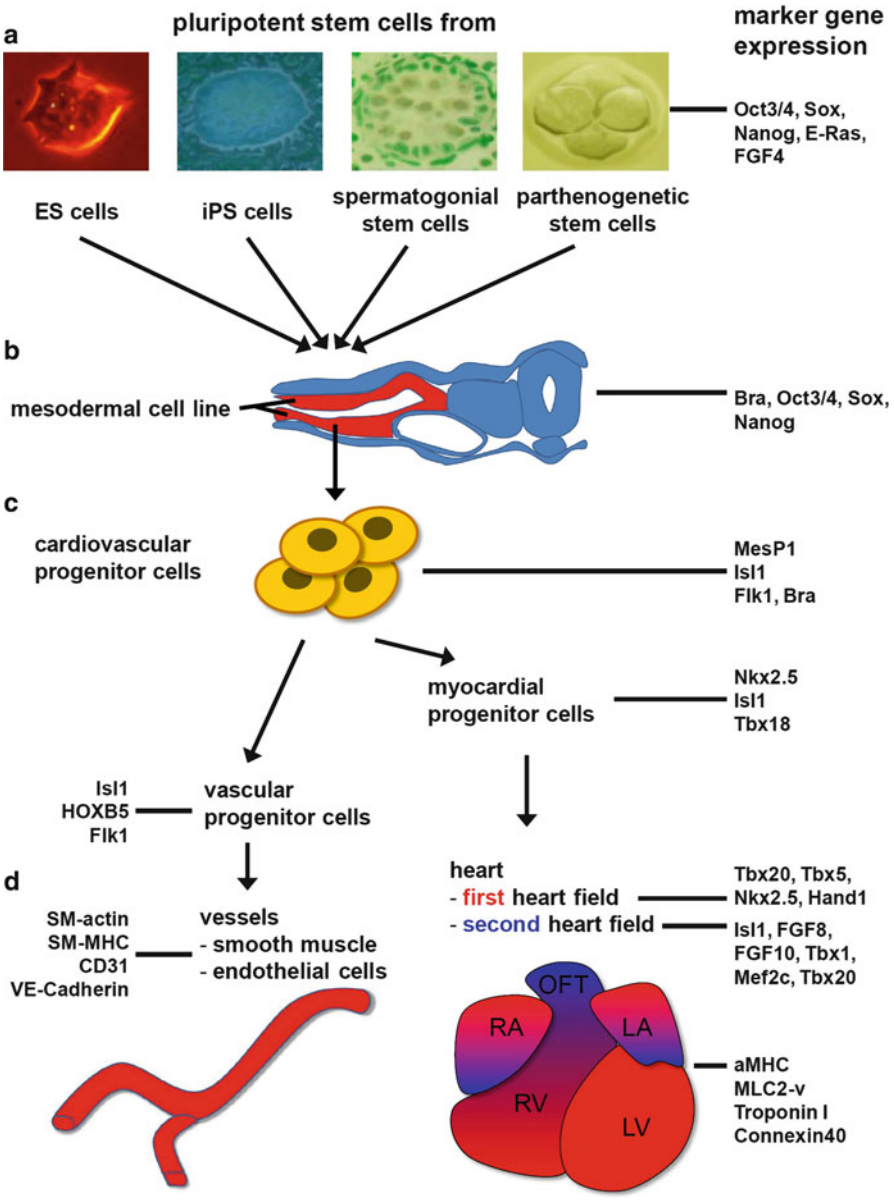


Fig. 11.1 Cardiovascular progenitors: Expression of marker genes during development from pluripotent stem cells to complete organs. (**a**) Pluripotent stem cells express the pluripotency markers Oct3/4, Sox, Nanog, E-Ras and FGF4 and can be derived from embryonic stem cells, induced pluripotent stem cells, spermatogonial stem cells or parthenogenetic stem cells. (**b**) All of the cell lines mentioned above are able to form a mesodermal cell lineage during stem cell differentiation. During this stage the pluripotency markers are downregulated and the first mesodermal transcription factor Brachyury (*Bra*) is expressed and again downregulated during further specification into the cardiovascular lineage. (**c**) The earliest marker of cardiovascular progenitor cells is the basic

for reprogramming or purification assays next to the markers described in the following sentences. MesP1 expressing cells therefore represent the first largely specific population of multipotent cardiovascular progenitor cells during embryogenesis that is able to form all components required to build up the heart. After creation of the cardiac crescent the cardiovascular stem cells get further specialized. Heart progenitors get committed irreversibly to a cardiac fate expressing the homeodomain transcription factors Islet1 (Isl1) and Nkx2.5 whereas the vascular progenitor cells are marked mainly by the expression of the homeobox transcription factor HOXB5 and the VEGF-receptor 2 (Flk1) (see Fig. 11.1) (Srivastava and Ivey 2006). Flk1 as well as the cell surface markers CD31 and VE-Cadherin expressed by fully differentiated endothelial cells can easily be used for antibody-based purification of vascular cells for further use in the means of therapeutical utilization by the methods described below. Moreover, an ES-cell derived cell population expressing Flk1 in a second wave and Brachyury was shown to have the ability not only to form endothelial cells but to work also as multipotent cardiovascular progenitors comparable to the MesP1 expressing cell line described above. This cell population showed cardiomyocytic, endothelial and vascular smooth muscular potential under cardiac cytokine stimulation (Kattman et al. 2006) and therefore could also be a suitable cell-source for transplantation.

Further cardiac development is based on two myocardial cell lineages that form the various regions of the heart. In particular, the cell lines can be roughly distinguished by their contribution to the formation of the left ventricle *(first heart field)* and the outflow tract *(second heart field)*, respectively (Kelly et al. 2001; Zaffran et al. 2004). The progenitor cells of the first heart field are mainly characterized by the expression of Nkx2.5, Tbx5 and Hand1 whereas this list seems not to be exhaustive at present. First heart field progenitors form both ventricles, atria and the atrioventricular canal. Cells expressing Isl1, FGF8, FGF10, Tbx1 and Mef2c originate from the mesodermal core of the pharyngeal arches and are attributed to the second heart field. They colonize the outflow tract and all other heart regions except of the left ventricle (see Fig. 11.1) (Buckingham et al. 2005; Kelly et al. 2001). The markers of the second heart field are explored quite well in contrast to those specific for the first heart field, but further investigation is required. For instance, Isl1 expressing cells can be used to generate smooth muscle, endothelial cells and cardiomyocytes but its knockout leads to a deficient development which is restricted to the outflow tract and right ventricle (Meilhac et al. 2004). In contrast, Nkx2.5 is expressed in both the first and second heart field but the Nkx2.5 knockout model lacks only the formation of Hand1 expressing cells which corresponds merely to the first heart field structures (Lyons et al. 1995). The Tbx5-knockout mouse shows quite similar defects even though not as pronounced.

It becomes clear that many overlapping expression patterns exist in parallel to each other and depending on the stage of development. And, without a doubt, not all (especially transient) expressions of certain cardiovascular markers have been detected, yet. Furthermore, till today it was not possible to isolate a specific progenitor cell for neither the first nor the second heart field as the markers mentioned above are preferably but not exclusively expressed in the respective precursor field

(Lam et al. 2009). The knowledge about the presently known specific markers expressed by the respective multipotent cardiovascular progenitor cells during the distinctive differentiation stages (in particular the $Flk1^+Bra^+$ (Kattman et al. 2006), the $MesP1^+$ (David et al. 2008a) and the $Isl1^+$ (Laugwitz et al. 2008) cell population) and the information about their developmental relevance is far from being complete today but nevertheless can already be used to identify, amplify and isolate the desired cell types in vitro from a pluripotent stem cell culture for generation of new cardiac tissue (David et al. 2009; Kattman et al. 2006; Müller et al. 2000; Wobus et al. 1997). To support this challenge various techniques have been introduced in recent years. These will be described in the following passages.

11.3 Derivation

The isolation of multipotent cardiovascular progenitor cells from a pluripotent stem cell culture has to face several obstacles. As described above, all conceivable types of precursor cells develop during the differentiation in vitro and can be identified by their specific gene expression program. Before being able to use these cells, e.g. for tissue engineering, they have to be cultured in a sufficient quantity and purified to avoid adverse reactions like teratoma formation (see Chap. 7) or malicious cardiac arrhythmias (Lee and Makkar 2004) after transplantation.

The differentiating ES cell culture only contains a few percent of beating cardiomyocytes (Xu et al. 2008) and therefore various attempts for increasing this number have been performed. The first approaches succeeded in stimulating the cardiogenesis by using exogenous stimulation methods (see Fig. 11.2 and Table 11.1). The addition of retinoic acid to the murine embryonic stem cell culture enhances the development of ventricular cardiomyocytes shown by increased expression of the αMHC and MLC2v genes (Wobus et al. 1997). A comparable effect can be reached by stimulation with ascorbic acid (Takahashi et al. 2003) or cultivation of ES cells under influence of a low frequency magnetic field (Ventura et al. 2005). A guided differentiation via pacemaker-like cardiomyocytes with expression of Cx40, Cx45 and typical transmembranous action potential can be attained by exposure of murine ES cells to endothelin-1. Regarding the human embryonic stem cell culture the increase of beating cardiomyocytes can be facilitated by coculturing of ES cells with endodermal END2 cells (Mummery et al. 2007) via yet undefined excreted serum factors or the addition of SB203580, a specific p38 MAP kinase inhibitor (Graichen et al. 2008). A complete review over the latest approaches for stimulating cardiogenesis with the help of external factors can be found in Table 11.1. These attempts all provided promising results but lacked the detailed analysis of an underlying signaling pathway and therefore await further investigation (see Fig. 11.2).

A more elegant way for generating increased numbers of cardiovascular progenitors in the pluripotent stem cell culture is *genetic forward programming* (see Fig. 11.2). Recently, David et al. have shown proof of principle for cardiovascular subtype specific programming of pluripotent stem cells. Via overexpression of early cardiovascular

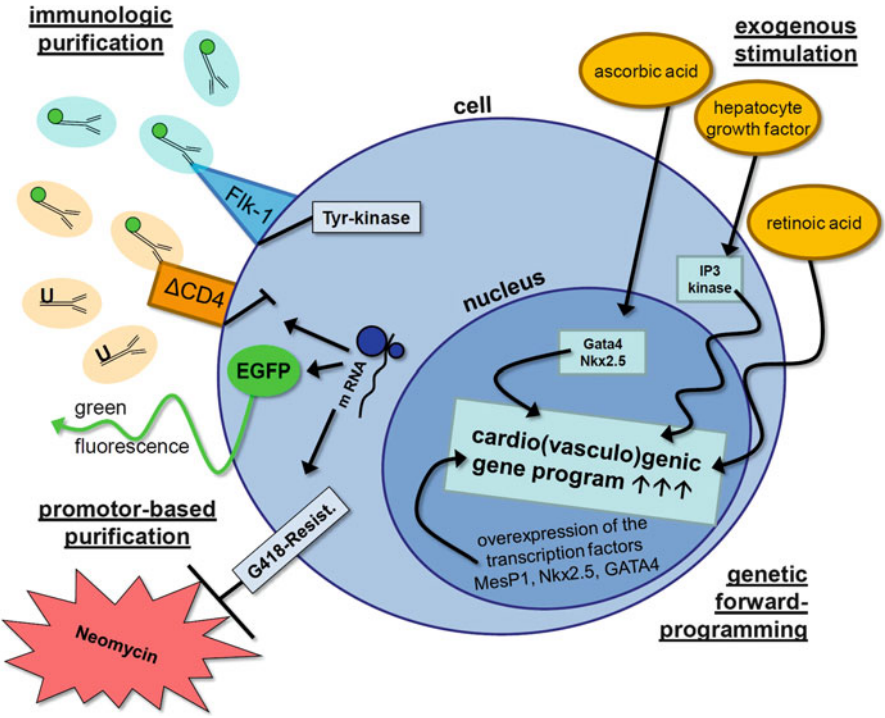


Fig. 11.2 Amplification and isolation of cardiovascular progenitor cells. *Exogenous stimulation*: The cardiovasculogenic program can be induced by addition of various agents (ascorbic and retinoic acid or hepatocyte growth factor) or the cultivation of the pluripotent stem cell culture in a low-frequency magnetic field. The underlying mechanisms are largely unknown here. *Genetic forward programming*: The differentiation process of pluripotent stem cells can be driven towards a cardiovascular fate via overexpression of selected cardiac (Nkx2.5) and cardiovascular (MesP1) transcription factors under control of the cytomegalovirus promotor. *Immunologic purification*: Cardiovascular progenitor cells can be purified from the differentiating stem cell culture via antibody-based fluorescence-activated or magnetic cell sorting. Antibodies can either bind to cardiovascular-specific endogenously expressed (Flk1) or transgenetically (promotor-based) expressed cell surface markers (ΔCD4). *Promotor-based purification:* The expression of structural proteins under control of specific cardiac and cardiovascular gene promotors facilitates the purification of cardiac or cardiovascular (progenitor) cells via magnetic cell sorting (ΔCD4), fluorescence activated cell sorting (EGFP) or antibiotic selection (G418-resistance)

transcription factors like Nkx2.5 (under control of the cytomegalovirus (CMV) promoter) the process of cardiogenesis could be induced in murine ES cells (David et al. 2009). Forced expression of this cardiac key gene leads to increased numbers of differentiated beating ventricular cardiomyocytes without affecting vascular progenitors in vitro whereas MesP1 overexpression causes an enhancement of vascular development shown by an increased number of electrophysiologically detectable early/intermediate type cardiomyocytes and spontaneously sprouting endothelial structures in the culture dish (David et al. 2008a, 2009). This is a strong sign for MesP1

Table 11.1 Exogenous induction of cardiovascular development in embryonic stem cells

Authors	Agent/method	Cell type	Effects
Wobus et al. (1997)	Retinoic acid	Mouse ES cells	Enhanced development of ventricular cardiomyocytes, up regulated MLC2v- and myosin heavy chain expression
Paquin et al. (2002)	Oxytocin	P19 embryonic stem cells	Increased number of beating cell colonies, up regulated ANP- and myosin heavy chain expression
Takahashi et al. (2003)	Ascorbic acid	Mouse ES cells	Increased number of alpha myosin heavy chain-positive cells, up regulated expression of GATA4, beta-MHC
Gassanov et al. (2004)	Endothelin	Mouse ES cells	Increased number of pacemaker-like cardiomyocytes, confirmed by electrophysiology (If) and immunostaining (Cx40, Cx45)
Ventura et al. (2004)	Butyric and retinoic mixed ester of hyaluron	Mouse ES cells	Increased number of spontaneously beating cardiomyocytes, up regulated expression of GATA4 and Nkx2.5
Ventura et al. (2005)	Extremely low frequency magnetic field	Mouse ES cells	Increased number of spontaneously beating cardiomyocytes, up regulated expression of GATA4 and Nkx2.5
Yuasa et al. (2005)	Noggin, inhibitor of BMP-signaling	Mouse ES cells	Increased beating EB incidence
Roggia et al. (2006)	Hepatocyte growth factor	Mouse ES cells	Increased number of beating embryoid bodies via PI3 kinase/Akt pathway, up regulated expression of GATA4 and Nkx2.5
Mummery et al. (2007)	Coculture with endodermal END2 cells	Human ES cells	Increased number of beating cardiomyocytes
Graichen et al. (2008)	SB203580, specific p38 MAP kinase inhibitor	Human ES cells	Increased cardiomyocyte yield via enhanced early mesoderm formation
Xu et al. (2008)	Prostaglandin I2	Human ES cells	Enhanced cardiogenic activity
Wiese et al. (2011)	Suramin	Mouse ES cells	Induction of differentiation into sinus node-like cells

lying upstream of Nkx2.5 in the molecular hierarchy for cardiovascular specification as the first-mentioned is acting on the level of cardiovascular progenitor cells whereas the influence of Nkx2.5 is restricted to the more specified ventricular cardiomyocyte progenitors (see Fig. 11.1). David et al. could detect a stimulation of cardiovascular development via MesP1-overexpression not only in early vertebrates but also in murine embryonic stem cells (David et al. 2008a). Subsequently, this key finding was confirmed by several other groups (Bondue et al. 2008; Lindsley et al. 2008). In the work of David et al. the underlying signaling pathway of MesP1 has been deciphered in detail. MesP1-overexpression and -knockdown experiments revealed a prominent function of MesP1 within a gene regulatory cascade causing Dkk1 mediated blockade of canonical Wnt-signalling. Independent evidence from chromatin immunoprecipitation, in vitro DNA binding studies, expression analysis in wild-type and MesP1/2 double knock-out mice and reporter gene assays confirmed the Dkk1 promoter as a direct target, activated by MesP1 protein (David et al. 2008a). This mechanism is supported by findings published by Lindsley et al. and Bondue et al. that detected a 50-fold and 1.5-fold upregulation of Dkk1-expression, respectively, caused by overexpression of MesP1 (Bondue et al. 2008; Lindsley et al. 2008).

Thus, it is evident that MesP1 is located at the top of the transcriptional network that controls cardiovascular differentiation by directly regulating the spatial and temporal expression of key cardiac transcription factors such as Nkx2.5, Tbx20, Hand2, Mef2c and indirectly by enhancing the transcription of Dkk1 (Bondue et al. 2008; David et al. 2008a; Lindsley et al. 2008; Wu 2008). Further attempts for genetic forward programming even though without a detailed related signaling pathway are summarized in Table 11.2.

Despite the progressing decryption of the molecular development of the heart and vessel formation, methods are not yet refined enough for scientists being able to direct pluripotent stem cell differentiation exclusively in the direction of cardiovascular development not to mention to generate pure cultures of specific differentiated myocardial, endothelial or smooth muscle cells with the help of gene technology methods. Therefore, to minimize the hazard of undifferentiated stem cells or improper cell types within the transplantable cell mass various purification methods have been introduced. The best established ways of isolating specific cells from a differentiating pluripotent stem cell culture are using the fluorescence activated or the magnetic cell sorting (FACS, MACS) (David et al. 2005; Kanno et al. 2004; Kattman et al. 2006; Müller et al. 2000). After labeling of the desired cells by antibodies binding to stage and cell type specific surface antigens the antibody-coupled cells can be detected and isolated by their fluorescence and magnetic properties, respectively (see Fig. 11.2). A suitable cell population for this approach are the $Flk1^{+}Bra^{+}$ cardiovascular progenitors described by Kattman et al. (Kattman et al. 2006) as Flk1 is endogenously expressed on the surface of these cells and therefore can be used for the methods described above. Merely the parallel expression of Flk1 in specified vascular progenitor cells (see Fig. 11.1) makes this marker less specific and thus can decrease the purity of the desired cardiovascular progenitor cell population. Possibly, the co-staining with Flk1- and CXCR4-antibodies may reduce this contamination (see Table 11.3) (Nelson et al. 2008). Unlike early cardiovascular or

Table 11.2 Induction of cardiovascular differentiation via genetical reprogramming

Authors	Genetic modification	Main affected signaling	Effect
Grepin et al. (1997)	GATA-4 overexpression	Nuclear target of inductive factors for precardiac cells	Accelerated cardiogenesis, increased number of terminally differentiated beating cardiomyocytes
Kanno et al. (2004)	NO overexpression	Not known	Accelerated cardiomyocyte differentiation, apoptosis of cells not committed to Cardiomyocyte-differentiation
Singh et al. (2007)	Chibby overexpression	Wnt/β-Catenin-pathway	Increased cardiac differentiation
David et al. (2009)	Nkx2.5 overexpression	Not known	Increased number of ventricular cardiomyocytes
David et al. (2009)	MesP1 overexpression	Wnt/β-Catenin-pathway	Increased number of early/intermediate type cardiomyocytes

Table 11.3 Purification of cardiovascular and cardiac progenitors by specific cell surface markers

Authors	Endogenous surface marker	Method	Cell type
Kattman et al. (2006)	Flk-1	FACS	Cardiovascular progenitor cells from murine ES cell culture
Nelson et al. (2008)	Flk-1, CXCR-4	FACS	Cardiopoietic lineage from murine ES cell culture

especially hematopoietic stem cells specified cardiac progenitors and differentiated cardiomyocytes do not express any known exclusive surface proteins that are accessible for antibody-based purification methods in their native state (Müller et al. 2000). For this reason the *promotor-based labeling* of cardiomyocytes was introduced (David et al. 2005; Kolossov et al. 2005). The transfection of a gene vector including a reporter gene (eGFP, ΔCD4 or Neomycin(G418)-Resistance) expressed under the control of a cardiac specific genetic promotor like MLC2v, αMHC (both specific for ventricular cardiomyocytes, (Kolossov et al. 2005; Müller et al. 2000; Zweigerdt et al. 2003)), Cx40 (cardiovascular progenitors, (David et al. 2008b)), or ANP (pacemaker cells, (Gassanov et al. 2004)) or the targeted knock-in of a reporter gene into the respective gene locus thereby facilitates the stable labeling of the desired cell type during a specific differentiation stage (see Fig. 11.2 and Table 11.4). The purification method is dependent on the used reporter gene. The intracellular expression of enhanced green fluorescent protein (EGFP) is only suitable for fluorescence activated cell sorting (FACS) whereas the intracellularly deleted (i.e. lacking any intracellular signal transduction) cluster of differentiation 4 (ΔCD4) can be

Table 11.4 Promotor-based purification methods of cardiac and cardiovascular cells from the embryonic stem cell culture

Authors	Promoter-based construct	Method	Cell type
Müller et al. (2000)	MLC2v-eGFP, CMV-enhancer	Percoll-gradient, FACS	Ventricular-like cardiomyocytes from murine ES cell culture
Zweigerdt et al. (2003) and Zandstra et al. (2003)	αMHC-Neomycin-resistance	Antibiotic selection	Beating cardiomyocytes
Gassanov et al. (2004)	ANP-eGFP	Suitable for FACS	Pacemaker-like cardiomyocytes from murine ES cell culture
David et al. (2005)	PGK-ΔCD4	MACS	Suitable for all cardiac-specific ΔCD4 expression constructs
Kolossov et al. (2005)	αMHC-eGFP	FACS	Atrial and pacemaker cardiomyocytes from murin ES cell culture
David et al. (2008b)	Cx40-eGFP	FACS	Cardiovascular progenitor cells from murine ES cell culture
Potta et al. (2010)	Acta3-PuroIRES2-EGFP	Antibiotic selection	Pacemaker-, atrial- and ventricular-like cardiomyocytes from murine ES cell culture

stained by FITC- (for FACS) or magnetic beat-coupled (for MACS) antibodies (see Fig. 11.2). A contrarian and not less elegant method is the negative selection with help of the neomycin-resistance gene. While the desired cells survive the treatment with the antibiotic G418 all needless cells are eradicated by this toxin (Zandstra et al. 2003; Zweigerdt et al. 2003).

Yet not all of the purification methods mentioned above are potentially suitable for the isolation of cells destined for the use in human beings. Using the antibiotic selection for example, it is not possible to gain stage-specific cardiovascular progenitor cells from the pluripotent stem cell culture as cardiovascular development is fast and the selection period in general takes several weeks (with the hazard of resistance and possible harmful effects of the antibiotic on terminally differentiated cells themselves) to guarantee a reliable purity of the desired cells (Klug et al. 1996; Zandstra et al. 2003; Zweigerdt et al. 2003). But also cytometry (FACS) cannot be seen as the gold standard of cell sorting as the cells get highly accelerated and irradiated by laser light as a side effect of the underlying method, which is likely to harm the purified cardiovascular cell types. Moreover, the green fluorescent protein EGFP used for labeling of the cells has been reported to bear pro-apoptotic properties (Liu et al. 1999) which would be a major hindrance for transplantation of such fluorescent cells into damaged myocardium. Therefore, magnetic cell sorting is currently regarded as the best method for a mild and time sparing cell purification. Using MACS up to 10^{11} cells can be analysed in about 1 h making it possible to separate large cell numbers and to identify even rare populations of cells. As described above, the use of transgenic cells expressing an intracellular truncated

human CD4 surface antigen thereby makes an immunogenic potential unlikely (David et al. 2005). But nevertheless it still has to be discussed whether the use of genetically modified cells in human beings can be performed at all, as transfected cells can carry the risk of malignant deterioration caused by the incidental integration of the transgene into the genome. Therefore, yet more efforts have to be made in future for further improvement of the compatibility associated with the purification methods.

11.4 Potential Applications for Therapies

Various applications for cardiac repair via pluripotent stem cell-derived cardiovascular progenitors have been proposed within recent years. With ischemic and dilative cardiomyopathy being on the list of the top cardiovascular diseases the generation of contractile myocardium presently is the main goal of current tissue engineering approaches (Tulloch et al. 2011; Zimmermann and Cesnjevar 2009). The transplantation of genetically and physiologically compatible myocardial tissue with an included and appropriate blood vessel supply will allow to compensate lost myocardial tissue – largely independent from its underlying prior injuring mechanism.

A further important application of cardiovascular tissue engineering is the generation of biological pacemaker and conducting cells (Gassanov et al. 2004; Shiba et al. 2009; Wiese et al. 2011) for patients suffering from sick-sinus-syndrome or disturbances of the conduction system like atrioventricular or bundle branch block. The implementation of those specified cells could prevent the necessity of electronic pacemaker implantations.

Minor possible employments for cardiovascular stem cells could be the generation of large vessels for use as vascular bypass grafts and heart valves as spare part for inoperative native cardiac valves (Srivastava and Ivey 2006). Presently, acceptable alternatives for both applications exist with the internal mammarian or radial arteries as bypass grafts and porcine or bovine pericardial valves for the respective use. Thus, the generation of these tissues currently is only a secondary goal.

However, comparable to other organ systems the ultimate object while working with pluripotent stem cell-derived progenitor cells will be the generation of a whole working organ system, i.e. in this case the artificial engineering of a working transplantable heart. But until this goal will be reached plenty of work still has to be done.

11.5 Conclusions and Future Development in Research

Till today a decent part of cardiovascular development has already been investigated. It is possible to generate, multiply, purify and maintain cardiovascular progenitors and their derivatives from pluripotent stem cells in vitro for further utilization, i.e.

to generate clinically applicable cardiac and cardiovascular replacement tissue. The knowledge and technology available today thereby provide the prerequisites necessary to let the vision of cardiac tissue engineering appear feasible within the next decades. For the remaining obstacles still lying in the way various approaches are already at least being investigated at the moment. Thus, the problem of immunologic rejection of graft tissue is most likely to be solved by the use of induced pluripotent stem cells. Furthermore, for the future development in research it can be expected that the ongoing decryption of the cellular signal transduction system in developing pluripotent stem cells will finally lead to the ability to exclusively direct stem cell differentiation into the cardiovascular fate. Ideally, the control of the molecular development should then be able by exogenous stimulation without using transgenic cells any more. This will then facilitate the generation of highly specific, pure and riskless transplantable cell populations.

References

Akins RE, Boyce RA, Madonna ML et al (1999) Cardiac organogenesis in vitro: reestablishment of three-dimensional tissue architecture by dissociated neonatal rat ventricular cells. Tissue Eng 5(2):103–118

Balsam LB, Wagers AJ, Christensen JL et al (2004) Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 428(6983):668–673

Bel A, Planat-Bernard V, Saito A et al (2010) Composite cell sheets: a further step toward safe and effective myocardial regeneration by cardiac progenitors derived from embryonic stem cells. Circulation 122(11 Suppl):S118–S123

Bondue A, Lapouge G, Paulissen C et al (2008) Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. Cell Stem Cell 3(1):69–84

Boyle A, Colvin-Adams M (2004) Recipient selection and management. Semin Thorac Cardiovasc Surg 16(4):358–363

Buckingham M, Meilhac S, Zaffran S (2005) Building the mammalian heart from two sources of myocardial cells. Nat Rev Genet 6(11):826–835

David R, Groebner M, Franz WM (2005) Magnetic cell sorting purification of differentiated embryonic stem cells stably expressing truncated human CD4 as surface marker. Stem Cells 23(4):477–482

David R, Brenner C, Stieber J et al (2008a) MesP1 drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling. Nat Cell Biol 10(3):338–345

David R, Theiss H, Franz WM (2008b) Connexin 40 promoter-based enrichment of embryonic stem cell-derived cardiovascular progenitor cells. Cells Tissues Organs 188(1–2):62–69

David R, Stieber J, Fischer E et al (2009) Forward programming of pluripotent stem cells towards distinct cardiovascular cell types. Cardiovasc Res 84(2):263–272

David R, Jarsch V, Schwarz F et al (2011) Induction of MesP1 by Brachyury(T) generates the common multipotent cardiovascular stem cell. Cardiovasc Res 92:115–122

Deindl E, Zaruba MM, Brunner S et al (2006) G-CSF administration after myocardial infarction in mice attenuates late ischemic cardiomyopathy by enhanced arteriogenesis. FASEB J 20(7):956–958

DESTATIS (2010) Cardiovascular diseases still leading cause of death in 2009. Press release from 18 Oct 2010, German Federal Statistical Office (371)

Eisenberg LM, Eisenberg CA (2006) Wnt signal transduction and the formation of the myocardium. Dev Biol 293(2):305–315

Engelmann MG, Theiss HD, Theiss C et al (2009) G-CSF in patients suffering from late revascularised ST elevation myocardial infarction: final 1-year-results of the G-CSF-STEMI Trial. Int J Cardiol 144:399–404

Gassanov N, Er F, Zagidullin N et al (2004) Endothelin induces differentiation of ANP-EGFP expressing embryonic stem cells towards a pacemaker phenotype. FASEB J 18(14):1710–1712

Gerecht-Nir S, David R, Zaruba M et al (2003) Human embryonic stem cells for cardiovascular repair. Cardiovasc Res 58(2):313–323

Graichen R, Xu X, Braam SR et al (2008) Enhanced cardiomyogenesis of human embryonic stem cells by a small molecular inhibitor of p38 MAPK. Differentiation 76(4):357–370

Grepin C, Nemer G, Nemer M (1997) Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. Development 124(12):2387–2395

Hunt SA (1998) Current status of cardiac transplantation. JAMA 280(19):1692–1698

Kanno S, Kim PK, Sallam K et al (2004) Nitric oxide facilitates cardiomyogenesis in mouse embryonic stem cells. Proc Natl Acad Sci USA 101(33):12277–12281

Kattman SJ, Huber TL, Keller GM (2006) Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. Dev Cell 11(5):723–732

Kelly RG, Brown NA, Buckingham ME (2001) The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. Dev Cell 1(3):435–440

Kessler PD, Byrne BJ (1999) Myoblast cell grafting into heart muscle: cellular biology and potential applications. Annu Rev Physiol 61:219–242

Kitajima S, Takagi A, Inoue T et al (2000) MesP1 and MesP2 are essential for the development of cardiac mesoderm. Development 127(15):3215–3226

Kleger A, Seufferlein T, Malan D et al (2010) Modulation of calcium-activated potassium channels induces cardiogenesis of pluripotent stem cells and enrichment of pacemaker-like cells. Circulation 122(18):1823–1836

Klug MG, Soonpaa MH, Koh GY et al (1996) Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. J Clin Invest 98(1):216–224

Kolossov E, Lu Z, Drobinskaya I et al (2005) Identification and characterization of embryonic stem cell-derived pacemaker and atrial cardiomyocytes. FASEB J 19(6):577–579

Kraehenbuehl TP, Ferreira LS, Hayward AM et al (2011) Human embryonic stem cell-derived microvascular grafts for cardiac tissue preservation after myocardial infarction. Biomaterials 32(4):1102–1109

Lam JT, Moretti A, Laugwitz KL (2009) Multipotent progenitor cells in regenerative cardiovascular medicine. Pediatr Cardiol 30(5):690–698

Larsen W (1998) Essentials of human embryology. Library of congress cataloging-in-publication data, 2nd edn. Churchill Livingstone, New York

Laugwitz KL, Moretti A, Caron L et al (2008) Islet1 cardiovascular progenitors: a single source for heart lineages? Development 135(2):193–205

Leal J, Luengo-Fernandez R, Gray A et al (2006) Economic burden of cardiovascular diseases in the enlarged European Union. Eur Heart J 27(13):1610–1619

Lee MS, Makkar RR (2004) Stem-cell transplantation in myocardial infarction: a status report. Ann Intern Med 140(9):729–737

Liao SY, Liu Y, Siu CW et al (2010) Proarrhythmic risk of embryonic stem cell-derived cardiomyocyte transplantation in infarcted myocardium. Heart Rhythm 7(12):1852–1859

Lin Q, Fu Q, Zhang Y et al (2010) Tumourigenesis in the infarcted rat heart is eliminated through differentiation and enrichment of the transplanted embryonic stem cells. Eur J Heart Fail 12(11):1179–1185

Lindsley RC, Gill JG, Murphy TL et al (2008) Mesp1 coordinately regulates cardiovascular fate restriction and epithelial-mesenchymal transition in differentiating ESCs. Cell Stem Cell 3(1):55–68

Liu HS, Jan MS, Chou CK et al (1999) Is green fluorescent protein toxic to the living cells? Biochem Biophys Res Commun 260(3):712–717

Lyons I, Parsons LM, Hartley L et al (1995) Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. Genes Dev 9(13):1654–1666

Maltsev VA, Rohwedel J, Hescheler J et al (1993) Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. Mech Dev 44(1):41–50
Meilhac SM, Esner M, Kelly RG et al (2004) The clonal origin of myocardial cells in different regions of the embryonic mouse heart. Dev Cell 6(5):685–698
Menasche P (2005) Skeletal myoblast for cell therapy. Coron Artery Dis 16(2):105–110
Menasche P, Hagege AA, Vilquin JT et al (2003) Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. J Am Coll Cardiol 41(7):1078–1083
Müller M, Fleischmann BK, Selbert S et al (2000) Selection of ventricular-like cardiomyocytes from ES cells in vitro. FASEB J 14(15):2540–2548
Mummery CL, Ward D and Passier R (2007) Differentiation of human embryonic stem cells to cardiomyocytes by coculture with endoderm in serum-free medium. Curr Protoc Stem Cell Biol Chapter 1: Unit 1F 2
Murry CE, Soonpaa MH, Reinecke H et al (2004) Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428(6983):664–668
Nelson TJ, Faustino RS, Chiriac A et al (2008) CXCR4+/FLK-1+ biomarkers select a cardiopoietic lineage from embryonic stem cells. Stem Cells 26(6):1464–1473
Neumayer HH (2005) Introducing everolimus (Certican) in organ transplantation: an overview of preclinical and early clinical developments. Transplantation 79(9 Suppl):S72–S75
Okano T, Yamada N, Okuhara M et al (1995) Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces. Biomaterials 16(4):297–303
Orlic D, Kajstura J, Chimenti S et al (2001) Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann N Y Acad Sci 938:221–229; discussion 229–230
Paquin J, Danalache BA, Jankowski M et al (2002) Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes. Proc Natl Acad Sci USA 99(14):9550–9555
Potta SP, Liang H, Winkler J et al (2010) Isolation and functional characterization of alpha-smooth muscle actin expressing cardiomyocytes from embryonic stem cells. Cell Physiol Biochem 25(6):595–604
Roggia C, Ukena C, Bohm M et al (2006) Hepatocyte growth factor (HGF) enhances cardiac commitment of differentiating embryonic stem cells by activating PI3 kinase. Exp Cell Res 313:921–930
Sadler T (1998) Medizinische Embryologie – Die normale menschliche Entwicklung und ihre Fehlbildungen 11th edition, Thieme, Stuttgart, Germany, ISBN-10: 3134466112, ISBN-13 978-3134466119 (german)
Saga Y, Miyagawa-Tomita S, Takagi A et al (1999) MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. Development 126(15):3437–3447
Saga Y, Kitajima S, Miyagawa-Tomita S (2000) Mesp1 expression is the earliest sign of cardiovascular development. Trends Cardiovasc Med 10(8):345–352
Schachinger V, Erbs S, Elsasser A et al (2006) Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 355(12):1210–1221
Shiba Y, Hauch KD, Laflamme MA (2009) Cardiac applications for human pluripotent stem cells. Curr Pharm Des 15(24):2791–2806
Singh AM, Li FQ, Hamazaki T, et al. (2007). Chibby, an Antagonist of the Wnt/{beta}-Catenin Pathway, Facilitates Cardiomyocyte Differentiation of Murine Embryonic Stem Cells. Circulation 115:617–626
Singla DK, Long X, Glass C et al (2011) iPS cells repair and regenerate infarcted myocardium. Mol Pharm 8:1573–1581
Soonpaa MH, Koh GY, Klug MG et al (1994) Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science 264(5155):98–101
Srivastava D, Ivey KN (2006) Potential of stem-cell-based therapies for heart disease. Nature 441(7097):1097–1099
Strauer BE, Brehm M, Zeus T et al (2002) Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 106(15):1913–1918
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676

Takahashi T, Lord B, Schulze PC et al (2003) Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. Circulation 107(14):1912–1916
Theiss HD, Brenner C, Engelmann MG et al (2010) Safety and efficacy of SITAgliptin plus GRanulocyte-colony-stimulating factor in patients suffering from acute myocardial infarction (SITAGRAMI-trial) – rationale, design and first interim analysis. Int J Cardiol 145(2):282–284
Tulloch NL, Muskheli V, Razumova MV et al (2011) Growth of engineered human myocardium with mechanical loading and vascular coculture. Circ Res 109(1):47–59
Ventura C, Maioli M, Asara Y et al (2004) Butyric and retinoic mixed ester of hyaluronan. A novel differentiating glycoconjugate affording a high throughput of cardiogenesis in embryonic stem cells. J Biol Chem 279(22):23574–23579
Ventura C, Maioli M, Asara Y et al (2005) Turning on stem cell cardiogenesis with extremely low frequency magnetic fields. FASEB J 19(1):155–157
Wiese C, Nikolova T, Zahanich I et al (2011) Differentiation induction of mouse embryonic stem cells into sinus node-like cells by suramin. Int J Cardiol 147(1):95–111
Wobus AM, Kaomei G, Shan J et al (1997) Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. J Mol Cell Cardiol 29(6):1525–1539
Wollert KC, Meyer GP, Lotz J et al (2004) Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. Lancet 364(9429):141–148
Wu SM (2008) Mesp1 at the heart of mesoderm lineage specification. Cell Stem Cell 3(1):1–2
Xu XQ, Graichen R, Soo SY et al (2008) Chemically defined medium supporting cardiomyocyte differentiation of human embryonic stem cells. Differentiation 76(9):958–970
Yamanaka S (2007) Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell Stem Cell 1(1):39–49
Yuasa S, Itabashi Y, Koshimizu U et al (2005) Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. Nat Biotechnol 23(5):607–611
Zaffran S, Kelly RG, Meilhac SM et al (2004) Right ventricular myocardium derives from the anterior heart field. Circ Res 95(3):261–268
Zandstra PW, Bauwens C, Yin T et al (2003) Scalable production of embryonic stem cell-derived cardiomyocytes. Tissue Eng 9(4):767–778
Zaruba MM, Theiss HD, Vallaster M et al (2009) Synergy between CD26/DPP-IV inhibition and G-CSF improves cardiac function after acute myocardial infarction. Cell Stem Cell 4(4):313–323
Zimmermann WH, Cesnjevar R (2009) Cardiac tissue engineering: implications for pediatric heart surgery. Pediatr Cardiol 30(5):716–723
Zweigerdt R, Burg M, Willbold E et al (2003) Generation of confluent cardiomyocyte monolayers derived from embryonic stem cells in suspension: a cell source for new therapies and screening strategies. Cytotherapy 5(5):399–413

Chapter 12
Neural Stem Cells

Yoko Arai, Wieland B. Huttner, and Federico Calegari

Abstract Neural stem cells are the source of all neurons, astrocytes and oligodendrocytes of the central nervous system. While the vast majority of neural stem cells are consumed during embryonic development, a subpopulation persists in specialized regions of the adult mammalian brain where addition of cells, notably neurons, continues throughout life. The significance and physiological role of adult neurogenesis are still debated but it is generally believed that neural stem cells may be used to establish novel therapies for certain neural pathologies. In this chapter we describe the main features of neural stem cells during embryonic development and adulthood as well as the key mechanisms known to influence their proliferation versus differentiation. We then discuss the current views on the function of adult neurogenesis and the first attempts to use neural stem cells in therapy. Since the focus of this book is on regenerative medicine, we will mainly describe neural stem cells of mammalian organisms and briefly mention studies on other phyla only if particularly relevant.

Y. Arai, Ph.D.
Alessandra Pierani Group, Genetics and Development of the Cerebral Cortex,
Institute Jacques-Monod CNRS UMR 7592, Université Paris Diderot,
Bâtiment Buffon, 15 rue Hélène Brion 75205, Paris, Cedex 13, France

W.B. Huttner, M.D. (✉)
Max Planck Institute of Molecular Cell Biology and Genetics,
Pfotenhauerstrasse 108, D-01307 Dresden, Germany
e-mail: huttner@mpi-cbg.de

F. Calegari, Ph.D.
DFG-Center and Cluster of Excellence for Regenerative Therapies,
Dresden University of Technology,
c/o Max Planck Institute of Molecular Cell Biology and Genetics,
Pfotenhauerstrasse 108, D-01307 Dresden, Germany

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_12,

Abbreviations

GABA	γ-aminobutyric acid
AP	anterior-posterior
AraC	arabinosyde-C
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
BrdU	bromodeoxyuridine
CSL	CBF1/RBPJk/*Supplessor of hairless*/Lag1
CNS	central nervous system
CDK	cyclin-dependent kinase
Dnmts	DNA methyltransferases
DV	dorso-ventral
EGF	epidermal growth factor
FGF2	fibroblast growth factor 2
HATs	histone acetylases
HDACs	histone deacetylases
HIF-Ia	hypoxia-inducible factor Iα
INM	interkinetic nuclear migration
miRNAs	microRNAs
NSC	Neural stem cells
Ngn	neurogenin
NICD	notch receptor
RA	retinoic acid
Shh	sonic hedgehog
SGZ	subgranular zone
SVZ	sub-ventricular zone
VZ	ventricular zone

12.1 Embryonic Neural Stem Cells, Their Lineage and Characteristics

Regenerative medicine for intractable brain disease benefits from the remarkable progress of neuroscience research. The aim and hope of regenerative medicine is the recovery or replacement of diseased cells and tissue in patients by application of *in vitro* developed cells or tissue recapitulating *in vivo* brain development (Ringe et al. 2002; Shastri 2006). Advantages of therapies using neural stem cells (NSC) are to regenerate organs without rejection by implanting regenerated cells into damaged organs and to supply nerve nutrition factors to support unhealthy brain cells (Okano 2002). These therapies are actively progressing together with the accumulation of results on the properties of NSC obtained by more fundamental neuroscience research (Okano and Sawamoto 2008). Therefore, it is important to understand the basic process of brain development. In this chapter, as a foundation for regenerative

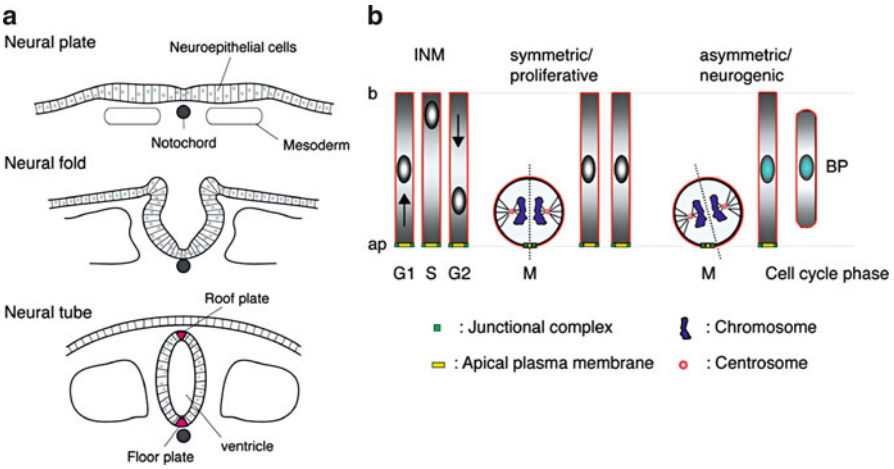


Fig. 12.1 Neural tube formation (a) and cell division of neuroepithelial progenitors (radial glial cells) (b). (**a**) At the neural plate stage, ectoderm overlying the notochord at the midline thickens to form the neural plate. At the neural fold stage, the neural plate invaginates to form the neural tube. At the neural tube stage, floor plate and roof plate cells become evident (*red*). (**b**) Interkinetic nuclear migration (*INM*) during the different phases of the cell cycle and symmetric *versus* asymmetric division of neuroepithelial cells. During M phase, the cleavage plane (*dotted line*) of apical progenitors bisects the apical domain for symmetric/proliferative division, whereas it bypasses this domain for asymmetric/neurogenic division, which generates a neuron (not illustrated) or a neuronally committed basal progenitor (*BP*). *ap* apical surface, *b* basal lamina

therapy, the development of the mammalian central nervous system (CNS) is described, particularly how multipotency of NSC is maintained and specification of differentiated cells is acquired. From a developmental point of view, embryonic NSC will form all neural cell types of the adult brain including the adult NSC.

12.1.1 Stem Cell Niche

NSC, collectively, can be regarded as multipotent progenitor cells and have (1) self-renewal capacity and (2) the potential to give rise to all neural cell types: neurons, astrocytes and oligodendrocytes. NSC are highly polarized epithelial cells, that is, the neuroepithelial cells that form the neural tube upon invagination of the neural plate during the process of neurulation (Fig. 12.1a). Neuroepithelial cells are arranged in a single layer of cells that forms the ventricular zone (VZ) (BoulderComm 1970). The VZ, whose apical side faces the ventricles and whose basal side faces the basal lamina, is colonized by blood vessels (Bautch and James 2009; Götz and Huttner 2005). This environment provides "stem cell niche"–like features to the neuroepithelial cells during development. Specifically, the ventricles are filled with lipoprotein- and membrane particle-rich cerebrospinal fluid, and the basal lamina is

a rich source of extracellular molecules including morphogens and growth factors (Raballo et al. 2000; Vaccarino et al. 1999a) (for more details, see Sect. 12.3). NSC are exposed to this environment through receptor interactions and endocytosis (Logan and Nusse 2004). In addition, the neurovascular communication is an important factor for the stem cell niche (Bautch and James 2009). Many molecules are supplied to NSC *via* blood vessels. At early stages of embryogenesis, endothelial cells, surrounding perivascular cells and neural cells interact and form "neurovascular units", which will be a basic unit of the future blood-brain barrier (Lok et al. 2007). Oxygen (O_2) is one of the important chemicals in the regulation of the NSC fate (Panchision 2009). In the mammalian CNS, the O_2 partial pressure (pO_2) is much lower than in air (20.8%) (Erecińska and Silver 2001; Panchision 2009). At such low pO_2, hypoxia-inducible factor Iα (HIF-Ia) facilitates signal transduction pathways stimulating self-renewal of NSC, whereas high pO_2 degrades HIF-Ia to promote neurogenesis (Gustafsson et al. 2005) and gliogenesis (Pistollato et al. 2007). Taken together, the ventricular fluid–, basal lamina– and blood vessel–based microenvironment of NSC provides important signals for the proliferation *versus* differentiation of these cells, and thus in the development of the CNS.

12.1.2 Characteristics of NSC In Vivo and In Vitro

In vivo, hallmarks of neuroepithelial cells in the CNS are (1) interkinetic nuclear migration (INM), the movement of cell nuclei from the apical luminal side (apical surface) to the basal side of the VZ (basal lamina) in concert with the progression of the cell cycle, and (2) cell polarity, with the plasma membrane of neuroepithelial cells being divided into two principal domains, an apical domain (apical plasma membrane) facing the ventricles and a basolateral domain which are separated from each other by junctional complexes (Fig. 12.1b). These characteristics have been well studied in the developing vertebrate CNS (Farkas and Huttner 2008; Götz and Huttner 2005; Kriegstein and Alvarez-Buylla 2009). At the beginning of brain development, neuroepithelial cells proliferate, generating two equivalent daughter cells in the VZ and increasing exponentially in number (Kageyama et al. 2008). At the onset of neurogenesis, intrinsic and extrinsic factors acting in concert control the production of neurons from neuroepithelial cells in a stepwise manner (Kriegstein and Götz 2003). Neuroepithelial cells transform into radial glial cells, collectively referred to as apical progenitors, that will progressively engage in differentiation. Apical progenitors change their division mode from a proliferative to a differentiative mode that leads to the production of neurons through the formation of so-called basal (or intermediate) neural progenitors (Götz and Huttner 2005; Haubensak et al. 2004; Hevner 2006; Noctor et al. 2004). In the developing cerebral cortex, basal progenitors form a second progenitor layer located basal to the VZ, the sub-ventricular zone (SVZ). In rodents, basal progenitors typically undergo one cell cycle and then generate two post-mitotic neurons which migrate out from the SVZ to appropriate neuronal layers (Hevner 2006). The sequential production of various types of neurons (neurogenesis) is then followed by gliogenesis (the birth of astrocytes and oligodendrocytes).

Region–specific *in vitro* NSC lines have been derived from fetal and adult CNS (Reynolds et al. 1992; Reynolds and Weiss 1992). Cells isolated from adult germinal regions are cultured in the presence of two growth factors, FGF2 (Fibroblast growth factor 2 or basic FGF) and EGF (epidermal growth factor) (see more detail in Sect. 12.3), and propagated in suspension culture as multicellular spherical aggregates termed "neurospheres" (Pollard et al. 2008). EGF, a known mitogen, and its receptors (EGFRs) are expressed in the CNS (Seroogy et al. 1995). EGF induces the proliferation of stem cells, which gives rise to neurospheres of undifferentiated cells that can differentiate into neurons, astrocytes and oligodendrocytes (Reynolds and Weiss 1996). Indeed, one NSC can expand more than 10^7 fold in the presence of EGF (Reynolds and Weiss 1996), as determined from clonal population analyses of embryonic and adult NSC *in vitro*. The simplified environment used in *in vitro* systems, though distinct from the physiological stimulation that NSC face in the niche, nonetheless provides an excellent tool to manipulate and dissect how cells undergo self-renewal and differentiation. In the following sections, mainly *in vivo* mechanisms will be reviewed, as the insight gained here provides an important basis to the development of regenerative applications.

12.1.3 Cell Cycle and Division

Fundamental biological mechanisms such as cell cycle and division are tightly linked to cell fate changes of NSC. Cell cycle kinetics of NSC are controlled by signaling pathways and cell-intrinsic determinants in order to obtain the appropriate balance between the growth of progenitors and their differentiation into neurons (Dehay and Kennedy 2007; Lukaszewicz et al. 2002; Ochiai et al. 2009; Ohnuma and Harris 2003; Ohnuma et al. 2001; Shimogori et al. 2004). The transition from one cell cycle phase to the next is controlled by the activation, via phosphorylation, of CDKs (cyclin-dependent kinases) that ensure that all cell cycle phases are executed in the correct order. Each CDK is dependent on a partner cyclin, which oscillates during the cell cycle to control its progression. Cyclin-CDK complexes form a driving force for the cell cycle (Ekholm and Reed 2000), which is inhibited by CKI (CDK inhibitor), a braking system in the cycle (Ohnuma et al. 2001) regulated by protein degradation (Nakayama and Nakayama 2006).

During neurogenesis, cell cycle progression of NSC is linked to INM (Fig. 12.1b) (Hayes and Nowakowski 2000). For M phase, the nucleus migrates to the apical side of the VZ where mitosis occurs, then the nucleus is translocated to the basal side during the G1 phase. DNA synthesis (S phase) occurs in the basal region of the VZ and is followed by the return of the nucleus to the apical side during the G2 phase (Kriegstein and Alvarez-Buylla 2009). Before overt neurogenesis in the cerebral cortex, NSC progression through the cell cycle is relatively fast, allowing self-amplification of NSC (Takahashi et al. 1995). During the progression of neurogenesis, the average cell cycle length increases, with lengthening notably in the G1 phase, as determined by cumulative BrdU labeling (Caviness et al. 1995; Takahashi et al. 1995), suggesting that the control of the G1 phase is a key step for the neurogenesis.

Activation of cyclin D/cdk4 and cyclin E/cdk2 complexes is an important step for the transition from G1 to S phase (Ekholm and Reed 2000), and accordingly the manipulation of G1/S transition by overexpression of cyclin D/cdk4 or cyclin E1 changes the cell fate of neural progenitors by shortening the G1 phase and total cell cycle length (Lange et al. 2009; Pilaz et al. 2009).

Before neurogenesis, NSC amplify their pool exponentially by symmetric proliferative division to generate two equivalent, proliferating daughter cells. At the beginning of neurogenesis, neurons and basal progenitors arise from asymmetric division of apical progenitors (radial glial cells), which allows their self-renewal as the other daughter cell remains an apical progenitor (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004, 2008). Symmetric divisions of basal progenitors generate neurons (Götz and Huttner 2005; Kriegstein and Alvarez-Buylla 2009). In the *Drosophila* CNS, asymmetric division is governed by various determinants, with notably the cleavage plane orientation during division being a critical fate determinant for the daughter cells (Doe and Skeath 1996). Specifically, a cleavage plane parallel to the apico-basal axis gives rise to symmetric division, while a cleavage plane perpendicular to this axis gives rise to asymmetric division (Doe and Skeath 1996). This orientation of cleavage planes results in the equal or unequal distribution of polarized cell fate determinants. In the developing mammalian CNS (Chenn and McConnell 1995), subtle variations in cleavage plane orientation are typically observed and the vast majority of cleavage planes are oriented parallel to the apico-basal axis, with an important feature being whether or not the tiny apical plasma membrane and the apical junctional complexes are bisected (Kosodo et al. 2004). Symmetric proliferative divisions bisect, and asymmetric neurogenic divisions bypass, this apical domain and thus have differential effects on its inheritance (Huttner and Brand 1997; Kosodo et al. 2004) (Fig. 12.1b). Lack of a precise orientation of the mitotic spindle perpendicular to, and of the cleavage plane parallel to, the apico-basal axis upon knock-down of Aspm (abnormal spindle-like microcephaly-associated) causes precocious neurogenesis (Fish et al. 2006, 2008). In contrast, deletion of the spindle regulator LGN results in the delamination and somal translocation of neural progenitors without significant consequences for neurogenesis (Konno et al. 2008; Morin et al. 2007). These findings indicate that precise control of spindle/cleavage orientation appears to be important for cell fate determination in a context-dependent manner.

Neuronal differentiation involves the specification of neural progenitors and neurons, and hence the appropriate alterations in gene expression controlled by cell-intrinsic and -extrinsic cues (Kriegstein and Götz 2003). After production of neurons, radial glial cells finally differentiate into glial cells such as astrocytes and oligodendrocytes. Therefore, in the rodent CNS, the following three steps occur sequentially; (1) expansion and self-renewal: symmetric proliferative and asymmetric divisions, respectively, of apical progenitors, (2) neurogenesis: differentiative divisions of apical and basal progenitors, (3) gliogenesis: differentiative divisions of neural progenitors (Qian et al. 2000). These aspects will be discussed in the following sections. First, the major intracellular players, complex intrinsic regulations involved in the determination of neural progenitors, will be discussed. Then, important

extracellular factors that influence neurogenesis and their respective signaling pathways will be reviewed. In the adult neurogenesis section, the generic aspects of gliogenesis will be addressed and regenerative applications will be discussed.

12.2 Cell-Intrinsic Factors in Neural Stem Cell Differentiation

To generate the variety of brain cells, NSC collectively have to be multipotent and provide the proper cell type at the proper time. The processes by which NSC generate a variety of functionally integrated neural progenitors and then neurons during embryonic development have been intensively studied, and the variety of neural progenitors is established by combinations of various cell-intrinsic factors: several types of transcription factors, receptors, ligands, cell cycle modulators and polarity proteins, etc (Kriegstein and Alvarez-Buylla 2009). A platform for neurogenesis is established by the coordinated functions of these players through signal transduction pathways.

12.2.1 Transcriptional Regulators

The major cell-intrinsic regulators are the basic helix-loop-helix (bHLH) transcription factors, which contribute to changing the characteristics of NSC over time during brain development: the self-renewal capacity, neurogenesis and gliogenesis (Kageyama and Nakanishi 1997). There are two types of bHLH genes: (1) the repressor-type bHLH genes, *Hes* genes, are mammalian homologues of *Drosophila hairy* and *Enhancer of split*, and (2) the activator-type bHLH genes, *Neurogenin* (*Ngn*), *Mash1* and *Math* genes, are mammalian homologues of *Drosophila* proneural genes *achaete–scute* complex and *atonal* (Kageyama et al. 2008). *Hes* genes not only regulate the maintenance of NSC but also promote gliogenesis in cooperation with Notch signaling (Lathia et al. 2008) (discussed below), while proneural genes including *Neurogenin* (*Ngn*), *Mash1* and *Math* are responsible for promoting neurogenesis (Ross et al. 2003).

12.2.1.1 Regulators of Maintenance and Proliferation

There are seven members in the *Hes* family (Kageyama et al. 2008). Among them, *Hes1*, *Hes3* and *Hes5* genes are highly expressed in the early stage of embryonic brain development to maintain NSC in an undifferentiated state and to inhibit their differentiation. Functional studies of *Hes* genes have been done using gene knock-out strategies. In *Hes1*:*Hes3*:*Hes5* triple knock-out mice, neuroepithelial cells prematurely differentiate into neurons as early as embryonic day (E) 8.5 when neuroepithelial cells in the wild-type are extensively self-renewing (Hatakeyama

et al. 2004). Importantly, in the triple knock-out mice, premature neurogenesis is already completed by E10 without generation of later-born cells, glia and ependymal cells, demonstrating that *Hes1*, *Hes3* and *Hes5* are also essential for fate determination of neural progenitors (Hatakeyama et al. 2004). Furthermore, *Hes1*:*Hes5* double knock-out neurospheres do not expand properly in contrast to wild-type (Ohtsuka et al. 1999). Hes genes are therefore important to regulate not only self-renewal and differentiation but also the multipotency of NSC for formation of neural tissue.

Interestingly, *Hes* genes also seem to have a role in tissue architecture, an important aspect with regard to tissue engineering. In *Hes1*:*Hes3*:*Hes5* triple knock-out embryos at E8.5, intracellular apical junctional complexes (adherens and tight junctions) and the basal lamina are disrupted and premature neurons scattered into the lumen and surrounding tissues (Hatakeyama et al. 2004). Therefore, *Hes* genes are essential for the structural integrity of the CNS.

12.2.1.2 Regulators of Differentiation

The activator-type bHLH genes, *Neurogenin 2* (*Ngn2*), *Mash1* and *Math* are expressed in neural progenitors that have a limited potential for proliferation (Fode et al. 2000). Upon expression of these bHLH genes, differentiation into neurons after repeated asymmetric cell division occurs as represented in Fig. 12.1b. Overexpression of the activator-type bHLH genes in NSC induces neuron-specific genes, and therefore these genes are known as proneural genes (Parras et al. 2002). Proneural genes also specify the neuronal subtypes. For example, in the dorsal part of cerebral cortex, *Ngn2* promotes generation of neurons and specifies glutamatergic pyramidal identity. In the ventral telencephalon, *Mash1* specifies γ-aminobutyric acid (GABA) –ergic inhibitory interneurons. Upon a change in progenitor identity as observed in *Ngn2* knock-out embryos, dorsal progenitors generate GABAergic instead of glutamatergic neurons, demonstrating the role of *Ngn2* in neuronal subtype specification (Parras et al. 2002; Perez et al. 1999). Thus, proneural genes are not only crucial for the generation of neurons but also for the acquisition of their proper identity.

12.2.1.3 Notch Signaling: Control Between Proliferation and Differentiation

Notch is a transmembrane receptor expressed by neural progenitors. Notch binds to its ligands Delta and Jagged, which are also transmembrane proteins and are also expressed by neural progenitors (Lathia et al. 2008). Once the Notch signaling pathway is activated, NSC maintain the proliferative and undifferentiated properties by induction of *Hes1* and *Hes5* genes which inhibit proneural gene expression. After receptor-ligand binding, the intracellular domain of the Notch receptor (NICD) is enzymatically cleaved by presenilin-1/γ-secretase and translocates to the nucleus

where the transcriptional repressor complex CSL (CBF1/RBPJk/*Suppressor of hairless*/Lag1) downregulates the transcription of *Hes* genes (Lathia et al. 2008). Nuclear NICD binds to CSL, which turns it into a transcription activator complex acting on chromatin remodeling factors to transcribe *Hes1* and *Hes5* genes (Artavanis-Tsakonas et al. 1999; Honjo 1996). Hes proteins in turn repress the transcription of proneural genes (*Ngn2*, *Mash1* and *Math3*), and their targets Delta and neuron-specific genes are not transcribed (Artavanis-Tsakonas et al. 1999). Conversely, in neuronally committed progenitors, expression of bHLH proneural genes promotes the transcription of neuron-specific genes and of *Delta* (Castro et al. 2006). Delta expressed from the committed progenitor activates Notch in the neighbouring cells to keep them in the undifferentiated state (Castro et al. 2006). This process is called "lateral inhibition" (Artavanis-Tsakonas et al. 1999). Therefore, Notch-Delta binding is the initial event for neuronal differentiation and a paradigmatic example of the crosstalk between neighboring cells.

12.2.1.4 Regulators of Regional Specificity

Higher brain functions rely on complex neuronal circuit formation between functionally specified regions. The fundamental regional organization of the brain is specified during early embryonic development by region-specific expression of transcription factors (Kriegstein and Götz 2003; Osumi et al. 2008). The transcription factor Pax6 is a key molecule to define the regional specificity of the CNS (Götz et al. 1998). Pax6 is specifically expressed in the dorsal telencephalon that gives rise to the neocortex (glutamatergic pyramidal neurons), the dorsal diencephalon that gives rise to the thalamus (thalamic neurons), the hindbrain which gives rise to the cerebellum (Purkinje neurons), and the dorsal brainstem (motor and sensory neurons) and spinal cord (motor and somatic neurons) (Simpson and Price 2002). In *Pax6* mutant animals, *Dlx1* and *Gsh2*, two genes identifying the ventral part of the telencephalon giving rise to basal ganglia (GABAergic neurons), are misexpressed in the dorsal cortex leading to a ventralization of the dorsal cortex (Stoykova et al. 1996; Toresson et al. 2000).

Pax6 is also important to control self-renewal (Arai et al. 2005) and differentiation (Götz et al. 1998) to generate neural progenitors (Estivill-Torrus et al. 2002; Warren et al. 1999). In the dorsal cortex, gain- and loss-of-function studies of Pax6 identified regulatory networks that control these processes. Pax6 acts by changing the combination of co-binding transcription factors in a dose-dependent manner (Sansom et al. 2009), which allows the regulation of expression of various downstream genes. Examples include (Sansom et al. 2009) (1) neural stem and progenitor maintenance of self-renewal capacity – Emx2, Sox9 and Hmga2, (2) cell cycle progression – G1 cyclin-dependent kinase (Cdk4) and Pten1, (3) neurogenesis – Ngn2 (bHLH) and Eomes/Tbr2, (4) chromatin binding –Cbx1 and Rnf2. Thus, Pax6 allows the integration of many biological pathways together with regional specificity cues.

12.2.2 Epigenetic Control in the Course of Differentiation

Other mechanisms that also contribute to cellular differentiation are epigenetic modifications. While the DNA sequence itself is generally conserved in somatic cells throughout the life of an organism, specific transcriptional regulation can be maintained by epigenetic mechanisms in individual progenitor cells and inherited by their differentiating progeny. Epigenetic modifications of cell type-specific genes contribute to cell-autonomous changes in NSC that regulate both neurogenic and gliogenic differentiation processes. Neurons, astrocytes and oligodendrocytes differentiate sequentially from NSC, and these programs can be recapitulated *in vitro*. NSC isolated form early embryonic stages generate, in terms of differentiated cells, predominantly neurons, while seemingly identical NSC isolated form later developmental stages generate predominantly astrocytes under the same culture conditions (Qian et al. 2000), suggesting that epigenetic mechanisms contribute to this change in the potential of NSC.

12.2.2.1 Histone Modifications and DNA Methylation

NSC fate is profoundly controlled by the spatiotemporal pattern of expression of transcription factors in concert with epigenetic modifications of their genome, including (1) histone modifications (acetylation, methylation, phosphorylation, ubiquitination and sumoylation) and (2) DNA methylation (Vincent and Van Seuningen 2009). When NSC self-renew, expression of lineage-specific genes is turned off and their chromatin is found in a "repressed" status as indicated by chemical modifications (deacetylation and methylation) of histones (Shi et al. 2008). These histone modifications occur most commonly on amino-terminal histone tails and provide a "histone code" that can be read by nuclear proteins to influence a multitude of cellular activities (Turner 2002).

The level of histone acetylation is regulated by the activity of histone acetylases (HATs) and histone deacetylases (HDACs) (Shi et al. 2008). It has been reported that HDAC-mediated transcriptional repression is essential for the maintenance and self-renewal of NSC (Sun et al. 2007). HDACs deacetylate lysine residues of histones resulting in chromatin condensation, which was shown to block access of transcription factors involved in neuronal differentiation (Shi et al. 2008). Tlx, a transcription factor essential for NSC proliferation, recruits HDACs onto *p21* (a CDK inhibitor) and *Pten* (a phosphatase and tumor suppressor gene) promoter regions to repress their expression, resulting in inhibition of neuronal differentiation (Sun et al. 2007), indicating that histone deacetylation by HDACs is a key step for gene silencing in NSC.

More recently, the potential role of histone methylation in CNS development has gained attention. Methylation of lysine residues of histone H3 and histone H4 has been observed in neuroepithelial cells, and variations in the degree of histone methylation (mono-, di- or trimethyl histones) has been implicated in neuronal differentiation

(Biron et al. 2004). Trimethyl histone H3 and monomethyl histone H4 have been found to be elevated in proliferating neural progenitors, while trimethyl histone H4 is enriched in differentiating neurons (Biron et al. 2004). Thus, an epigenetic program based on a highly dynamic regulation of histone lysine methylation seems to participate in the neural differentiation process.

The epigenetic status is also regulated by DNA methylation. The level of DNA methylation of the promoter region of a gene often reflects its state of repression. DNA methylation is a post-replicative modification of cytosine (C) that occurs predominantly within CpG dinucleotides (Rottach et al. 2009) and is catalyzed by DNA methyltransferases (Dnmts) (Robertson and Wolffe 2000). Dnmt1 null embryos show embryonic lethality (Li et al. 1992), and conditional Dnmt1 depletion in neural progenitors results in DNA hypomethylation and precocious astroglial differentiation (Fan et al. 2005), suggesting that the maintenance of DNA methylation is important for normal development and controls the timing of gliogenesis. Another two independently encoded DNA methyltransferase genes, *Dnmt3a* and *Dnmt3b*, are expressed in the CNS (Okano et al. 1999; Watanabe et al. 2002, 2006). While *Dnmt3b* is specifically expressed in neural progenitors, *Dnmt3a* is expressed in postmitotic neurons (Watanabe et al. 2006). *Dnmt3b* null embryos have multiple developmental defects, indicating an important role of DNA methylation in the initial steps of differentiation. *Dnmt3a* null embryos develop until 4 weeks after birth (Okano et al. 1999), and it has been suggested that Dnmt3a is required for the establishment of proper tissue-specific DNA methylation patterns.

Recently, a link between DNA methylation and cell type-specific gene expression was reported. DNA methylation itself is involved in the repression of *GFAP*, which is expressed in astrocytes (Takizawa et al. 2001). Interestingly, DNA methylation coupled to chromatin remodeling also plays a crucial role in regulating neuronal activity-dependent genes like *BDNF* (brain derived neurotrophic factor) (Martinowich et al. 2003). Demethylation of the *BDNF* promoter region was observed upon depolarization, releasing its repression and therefore allowing its expression in active neuronal networks. This again puts emphasis on the crucial role of epigenetic mechanisms for the regulation of factors involved in neuronal function, in this case plasticity (Martinowich et al. 2003).

12.2.2.2 Chromatin Remodeling

A role of chromatin-based epigenetic mechanisms in early neural development has been reported (Aigner et al. 2007; Lessard et al. 2007). Chromatin remodeling involves the effective shifting of nucleosome cores along the length of the DNA molecule, a process known as “nucleosome sliding”. Chromatin remodeling is accomplished, at least in part, by ATPase-containing complexes, referred to as ATP-dependent SWI/SNF-like chromatin remodeling complexes (Cheng et al. 2005; Strahl and Allis 2000). Mammalian NSC and proliferating progenitor cells express the complexes SWI2/SNF2-like ATPases together with BAF45a, a Krüppel/PHD domain protein, and BAF53a (Lessard et al. 2007). Conversely, when NSC

exit the cell cycle, the homologues BAF45b, BAF45c and BAF53b replace the respective subunits specific of proliferating NSC (Lessard et al. 2007). The combination of chromatin remodeling factors therefore seems to add another degree of complexity to the regulation of factors involved in the proliferation/differentiation fate choice of NSC.

12.2.3 MicroRNAs

MicroRNAs (miRNAs) are 20–25 nucleotide-long non-coding RNAs that negatively regulate the stability and translation of target mRNA (Ambros 2004; Bartel 2009). Approximately 70% of the known miRNAs are found in the brain (Du and Zamore 2005). miRNAs are expressed in a tissue-specific and developmentally regulated manner (Ambros 2004; Bartel 2009). A large fraction of miRNA genes are found within introns of transcripts generated by RNA polymerase II (Kim 2005). These primary transcripts of miRNAs (pri-miRNA) are first processed into 60–75 nucleotide-long hairpin-like precursors (pre-miRNAs) by the RNase III endonuclease. They are then exported to the cytoplasm where they are cleaved into mature miRNAs by Dicer, a cytoplasmic RNase III -type endonuclease. miRNA recognition of a target mRNA results in its decreased stability and translation and hence in reduced expression of the respective gene (Klein et al. 2005).

The deletion of Dicer1 causes embryonic lethality and loss of stem cell pools (Bernstein et al. 2003). However, conditional Dicer knock-out in the developing cerebral cortex does not impair the early expansion of NSC and the generation of basal progenitors but does result in a dramatic size reduction of the cerebral cortex and in a disruption of its neuronal layering (De Pietri Tonelli et al. 2008), indicating that miRNAs control, in particular, neuronal differentiation.

miRNAs are key regulators of stem cell biology in general, and of neural development in particular, and have been implicated in cell fate decisions based on their expression patterns, computationally predicted targets and overexpression analyses (Cao et al. 2006; Houbaviy et al. 2003; Smirnova et al. 2005; Suh et al. 2004). During neurogenesis, several neuronal miRNAs show linage-specific expression (Smirnova et al. 2005). miR9 and miR125 are expressed in the neural tube and found in both the germinal and the neuronal layers, whereas miR124 expression is predominantly observed in neurons, miR23 in astrocytes and miR26 and miR29 in both neurons and astrocytes (Cao et al. 2006; Smirnova et al. 2005). Overexpression of miR9 and miR124 in neuronal progenitors decreases astrocyte differentiation, while inhibition of miR9 alone or together with miR124 reduces the number of neurons (Smirnova et al. 2005), consistent with a role of neural miRNAs (miR9 and miR124) in neurogenesis (Cheng et al. 2009). Recently, *laminin γ1* and *integrin β1*, which are highly expressed in neural progenitors and repressed during neuronal differentiation, have been reported to be target genes of miR124 (Cao et al. 2007), thereby providing a possible mechanism for the miR124-induced alteration in neural progenitor proliferation. In addition, miR124 binds to the 3'UTR of SCP1 to antagonize

its anti-neural function (Visvanathan et al. 2007), thus acting on broader signal transduction pathways which may affect various cellular functions. Clearly, a comprehensive identification of miRNAs and their targets genes will provide important insight into the regulation of NSC proliferation *versus* differentiation.

12.3 Cell-Extrinsic Factors in Neural Stem Cell Differentiation

Neurogenesis is highly dependent on the proper environment, which affects cell behavior and identity. In other words, NSC proliferation and differentiation will be influenced by extracellular signals that convey information about growth conditions as well as positional information (Cayuso and Martí 2005). Extracellular signaling molecules, notably growth factors and morphogens, are key factors of the microenvironment in which NSC reside, the stem cell niche, and are crucial for coordinating CNS development. "Pattern formation is the mechanism by which initially equipotent embryonic cells proliferate and organize into an intricate spatial arrangement of diverse cell types" (Wolpert 1969).

12.3.1 Morphogens and Identity

In the developing brain, positional information along the anterior-posterior (AP) and dorso-ventral (DV) body axes is encoded by morphogens. Morphogens are secreted molecules that influence gene expression in a concentration-dependent manner. Morphogens are produced from sources called signal-organizing centers and diffuse to form a concentration gradient, which is then integrated by the receiving cells and will affect various cellular aspects including cell migration, organization and identity.

Shh (sonic hedgehog), a major morphogen, is a member of the Hh (hedgehog) family and the best studied ligand of this signaling pathway. Shh is produced in two ventral midline signaling centers, the floor plate of the neural tube and the underlying notochord, an axial mesodermal structure (Martí et al. 1995). To travel far (long-range activity) from its source along the DV axis, Shh requires an auto-processing event that releases an active, cholesterol-modified, N-terminal fragment (N-Shh) (Ingham and McMahon 2001). Graded Shh concentration along the DV axis in a ventral-high, dorsal-low profile allows the initial patterning of the progenitor domains within the ventral neural tube (Briscoe et al. 2000; Jessell 2000; Pierani et al. 1999). This gradient is converted into the intracellular expression of various homeodomain transcription factors that define progenitor domain identity (Briscoe et al. 2000). In the developing ventral spinal cord, five different types of post-mitotic neurons (four interneurons and one motor neuron) are generated from these progenitor domains (Briscoe et al. 2000; Pierani et al. 1999, 2001). Thus, Shh controls the generation of distinct post-mitotic neurons along the DV axis. With regard to the

field of regenerative medicine, for example spinal cord injury which typically is localized to a specific area and hence to neuronal subgroups, these findings provide crucial basic knowledge for designing appropriate therapeutic approaches.

BMPs (bone morphogenetic proteins), belong to the transforming growth factor β (TGF-β) family, and multiple BMPs are secreted from the roof plate and the dorsal neural tube (Liem et al. 1997). Several studies indicate that BMPs function in dorsal patterning of the spinal cord to antagonize the ventral patterning effect by Shh (Chesnutt et al. 2004; Liem et al. 1997; McMahon et al. 1998; Wine-Lee et al. 2004). BMPs bind to two families of receptor serine/threonine kinases, type I and II, and propagate the signal by phosphorylation of Smad proteins (Shi and Massagué 2003). Disrupting BMP signaling with the BMP antagonist Noggin affects particularly the dorsal neuron identity in the spinal cord where BMP acts as morphogen, and can be rescued by BMP4 exposure (Liem et al. 1997). The double knock-out of BMP receptors (BMPRIa and Ib) and knock-down of Smad4 by RNA interference result in loss of the dorsal-most phenotype (Chesnutt et al. 2004; Wine-Lee et al. 2004), thus adding further evidence for the role of BMPs in the establishment of the dorsal progenitor domain identity in the spinal cord.

The proper development of the CNS requires differentiation to proceed not only along the DV, but also the AP, body axis. FGFs (fibroblast growth factors), which have various functions in the cell biology of the NSCs (Mason 2007), are also involved in the AP "body plan" formation process. FGFs are monomeric ligands and activate FGF receptor (FGFR) tyrosine kinase (Mason 2007). FGF8 from the presomitic mesoderm is known to be important for the caudal body axis extension by controlling the proliferation in a "stem cell zone", composed of self-renewing progenitors, in the ridge of the caudal neural tube (Diez del Corral et al. 2002). FGF8-exposed progenitors differentiate into neurons only after neural tube closure and following exposure to retinoic acid (RA), produced from the somitic mesoderm surrounding the neural tube (Diez del Corral et al. 2003). Thus, FGF signaling is involved the maintenance of self-renewal and an undifferentiated state of progenitors, whereas RA promotes neurogenesis, with the interplay between these two factors governing the progression of neurogenesis along the AP axis.

12.3.2 Morphogens and Growth

Morphogens such as Shh and BMPs are not only involved in patterning but also influence the proliferation and survival of progenitors. Shh has a known mitogenic function (Dahmane et al. 2001; Ulloa and Briscoe 2007) as shown by gain- and loss-of-function studies analyzing the proliferation of neural progenitors in the CNS (Cayuso et al. 2006; Chiang et al. 1996; Ishibashi and McMahon 2002) including the cerebral cortex (Komada et al. 2008). In Shh signal-receiving cells, binding to Patched (Ptc) receptors (Ingham and McMahon 2001) releases the inhibition of the receptor Smoothened and activates downstream target genes involved in cell proliferation like Cyclin D and N-Myc through Gli activation (Jacob and Briscoe 2003;

Kenney et al. 2003; Kenney and Rowitch 2000; Ulloa and Briscoe 2007). Proliferation and cell cycle kinetics are affected in *Shh* conditional knock-out mice, which show an increase in cell cycle length (Komada et al. 2008). BMPs also control proliferation of NSC and progenitors either through their downstream targets (cyclin D1 and cdk4) (Hu et al. 2001) or Wnt canonical signaling pathways (Nusse et al. 2008). Transgenic embryos with a constitutively active BMPRIa (caBMPR1a) (see also Sect. 12.2.1) show a robust proliferation of neural progenitors at early stages, and the morphology of the neuroepithelium is severely altered, with the appearance of gyrus-like structures (Panchision et al. 2001), whereas constitutively active BMPRIb (caBMPR1b) promotes neurogenesis (Panchision et al. 2001). Furthermore, caBMPR1a induces expression of *Wnt1* and *Wnt3*, two mitogenic *Wnt* genes, indicating that the mitogenic effects of BMPs may depend on the mitogenic activity of Wnt. Induction by the BMP pathway of *Msx1*, a known inhibitor of proneural genes (see Sect. 12.2.1.2), could also contribute to the mitogenic activity of BMPs (Liu et al. 2004).

Wnt ligands form a family of secreted glycoproteins related to *Drosophila* Wingless and participate in multiple developmental events during embryogenesis (Logan and Nusse 2004). Wnt effects are pleiotropic and include mitogenic stimulation, cell fate specification and differentiation. Wnt signaling through its receptors (Frizzled) leads to the translocation of β-catenin to the nucleus to form a transcriptional complex with TCFs (T-cell factor), a pathway called canonical Wnt pathway (Logan and Nusse 2004). Evidence for the control of proliferation by the canonical Wnt pathway has been obtained by gain- and loss-of-function approaches for Wnts (Lange et al. 2006) and β-catenin (Chenn and Walsh 2003; Machon et al. 2003; Megason and McMahon 2002). In the developing CNS, the most prominent member of the Wnt family, Wnt1, is expressed at the dorsal midline along the entire AP axis (Gavin et al. 1990; Parr et al. 1993), whereas β-catenin is expressed ubiquitously in the VZ, with relatively strong immunoreactivity at the apical, luminal side of the VZ (Chenn and Walsh 2003; Megason and McMahon 2002). Ectopic expression of Wnt1 and Wnt3a in transgenic mice causes overgrowth of the neural tube at the dorsal midline (Dickinson et al. 1994; Megason and McMahon 2002). Wnt7a and 7b are also important for cell proliferation in the VZ and SVZ of the cerebral cortex (Viti et al. 2003). Consistent with the role of Wnts in stimulating proliferation, expression of constitutively active β-catenin increases progenitor proliferation and decreases neurogenesis (Megason and McMahon 2002; Zechner et al. 2003) (Chenn and Walsh 2003). In contrast, ablation of β-catenin causes a reduction of tissue mass (Zechner et al. 2003). These Wnt/β-catenin-mediated mitogenic effects occur through downstream target genes (cyclin D, c-Myc and connexin43) which regulate G1/S transition of the cell cycle (He et al. 1998; Lange et al. 2006; Logan and Nusse 2004; Shtutman et al. 1999; Tetsu and McCormick 1999).

FGF2 (basic FGF) is also known to stimulate the proliferation of progenitors in primary cultures isolated from embryonic cerebral cortex by shortening cell cycle length (specifically G1) (Lukaszewicz et al. 2002). FGF2 is highly expressed in the developing VZ and SVZ, and knock-out mice show a reduction in proliferation, VZ volume and total cell number during development. Furthermore, in adult knock-out mice, the numbers of pyramidal neurons and glial cells are equally reduced (Korada

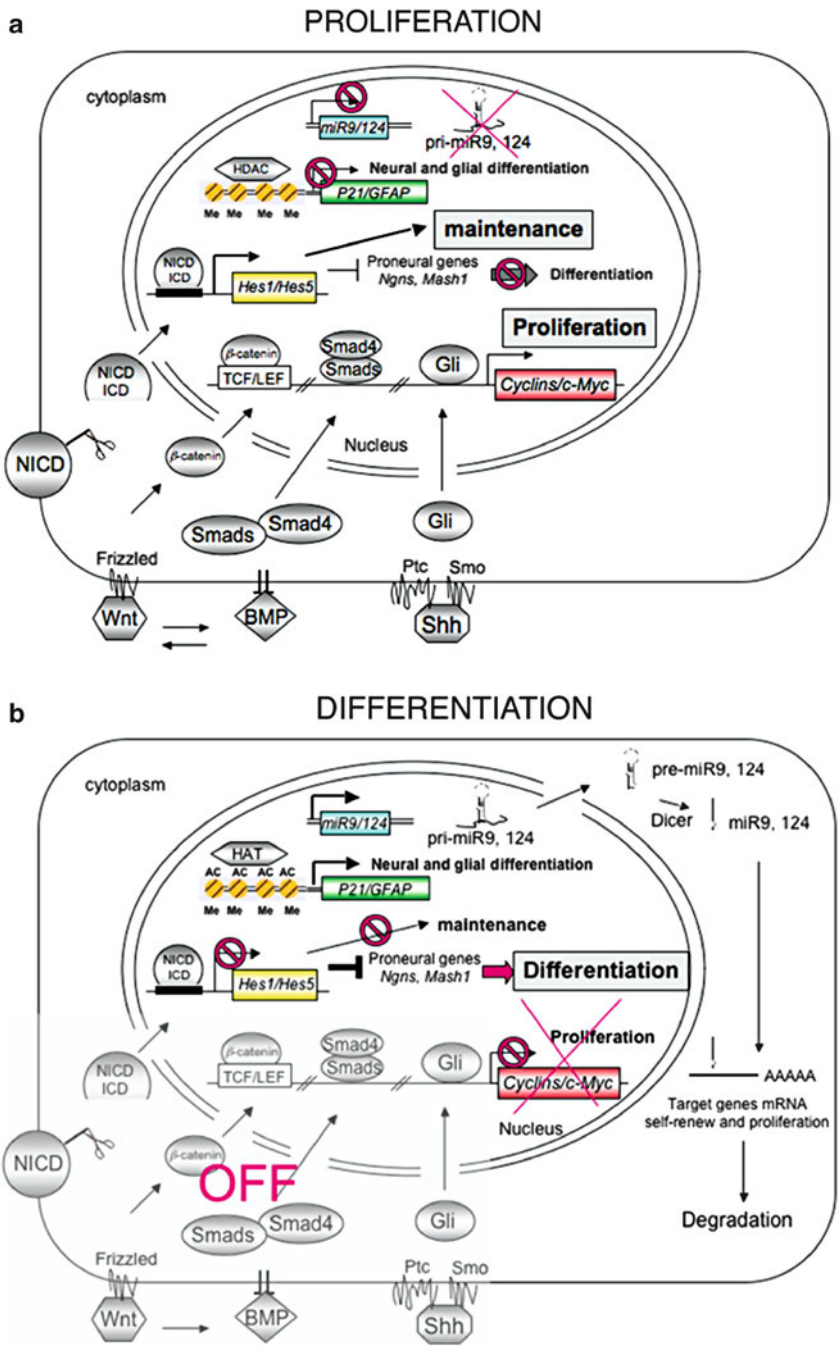


Fig. 12.2 Cross-talk between cell-intrinsic and -extrinsic factors in neural progenitors. Schematic representation of the various signaling pathways that affect the fate of NSC; (**a**) maintenance of NSC proliferation and an undifferentiated state, (**b**) differentiation of progenitors

et al. 2002; Raballo et al. 2000; Vaccarino et al. 1999b), consistent with FGF2 being a potent mitogen *in vivo*. Interactions between FGFs and the Notch signaling pathway have been described (Yoon et al. 2004). Progenitors isolated from the VZ in the presence of FGF2 increase *Notch1* and decrease *Delta1* expression (Yoon et al. 2004), suggesting that at least part of the FGF activity is mediated through the Notch pathway.

Some FGFs are produced locally by signal-organizing centers, in particular in the anterior medial part of the cerebral cortex. Conditional inactivation of FGF8 in the cortex results in a significant reduction of cortical size and mitotic index, and in robust apoptosis (Storm et al. 2006). In contrast to FGF2, FGF8 promotes differentiation and cell cycle exit (Borello et al. 2008). The various FGFs and their receptors play pivotal roles in the regulation of proliferation and the genesis of cortical neurons and glia. EGF, often used as a supplement in cell culture to sustain proliferation, behaves like a mitogen for late embryonic and adult NSC (Reynolds et al. 1992; Reynolds and Weiss 1992).

To conclude, many extrinsic factors are required, in appropriate spatio-temporal patterns, for proper CNS development. Most of them act as either mitogens or morphogens involved in proliferation control and providing positional cues to neural progenitors, which will affect the timing of neurogenesis and neuronal identity.

Many factors, some extrinsic other intrinsic, orchestrate the proliferation *versus* differentiation fate choice of NSC (Fig. 12.2a, b). The underlying integration of information is amazingly complex and occurs at various regulatory levels. Understanding normal development and neural stem cell biology will bring us closer to designing the future tools for regenerative medicine.

12.4 Adult Neural Stem Cells

12.4.1 Introduction: A Historical Perspective

It was 1906 when for the first time two scientists shared the Nobel award. These were Bartolomeo Camillo Golgi for developing the silver impregnation reaction (*la reazione nera*) and Santiago Ramon y Cajal for the demonstration, using Golgi's method, that the brain is made of contiguous, individual nerve cells (*the neuron theory*) (Lopez-Munoz et al. 2006).

The works of Golgi and Cajal represented a fundamental leap to start revealing the extraordinary complex cytoarchitecture of the adult brain, which, on the other hand, made it difficult to consider that this organ may undergo remodeling during adult life. The vision of the adult brain as a static and unmodifiable structure was also corroborated by clinical and functional observations, at least those that could be made with the tools available at the time. For example, it was known that patients with injuries to the central nervous system (CNS) or neurological pathologies had very little, if any, possibility of recovery and that even a minor lesion to the CNS may lead to major deficits in its function. Therefore, the scientific community was

confident that CNS plasticity must be terminated after development, as summarized by Cajal himself in an often-cited statement: "*Once development was ended*, *the fonts of growth and regeneration of the axons and dendrites dried up irrevocably*. *In adult centres*, *the nerve paths are something fixed and immutable*: *everything may die*, *nothing may be regenerated*. *It is for the science of the future to change*, *if possible*, *this harsh decree*" (Ramon y Cajal 1913)

And indeed, science has changed this view. The first evidence of adult neurogenesis came nearly 50 years later, after the characterization of tritiated thymidine and autoradiography as a system to identify newly synthesized DNA and, thus, cells undergoing S phase in living tissues (Firket and Verly 1958). To appreciate the impact of this revolutionary approach, it should be considered that this was the first time that scientists had the opportunity to retrospectively analyze the time of origin, lineage and migration of cells in living organisms. This was due to the fact that the radiolabeled compound (1) is rapidly metabolized after administration, which allows pulse-chase labeling of cells, and (2) is irreversibly incorporated in the DNA allowing the identification of daughter cells long after their mother had concluded S phase.

It was Joseph Altman to first use this approach to investigate the possibility of adult neurogenesis by administering the radiolabeled compound to the injured brain followed, 1 month later, by its detection in cells that were morphologically classified as neurons (Altman 1962). Interestingly for stem cell biologists today, Altman did not interpret this finding as to indicate that neurons may be activated to enter S phase and, eventually, divide. Rather, he correctly concluded that "…*new neurons may arise from non differentiated precursors*, *such as ependymal cells*. *After multiplication*, *such embryonic cells could differentiate and thus add new neurons to the existing population*." (Altman 1962). Notably, the identity of ependymal cells as true neural stem cells is still debated at the time of writing (Chojnacki et al. 2009).

Altman also made similar observations in other brain regions. However, since the neurons observed were, admittedly, very few and no technique was yet available to ascertain their identity, the scientific community tended to consider adult neurogenesis unconvincing or, in the most benevolent cases, negligible and unimportant. This view did not change even after the advent of electron microscopy and a more reliable identification of newborn adult neurons by ultrastructural analysis, as first established by Michael Kaplan (Kaplan and Hinds 1977).

Only the combination of S phase radiolabeling, ultrastructural analyses and electrophysiology undertaken by Fernando Nottebohm in the 1980s could finally provide proof of adult neurogenesis (Nottebohm 1985). This is not to say that adult neurogenesis was accepted as a reality for Nottebohm's studies were limited to canaries, a species of songbirds known to undergo seasonal brain remodeling, which suggested that adult neurogenesis was, if at all, limited to few and rare species. Nevertheless, Nottebohm's work acted as a catalyzer for new investigations in mammals, which were gaining momentum also due to the use of the thymidine-analogue bromodeoxyuridine (BrdU), which, in contrast to radio-labeling, allowed immunodetection together with established molecular markers of neurons.

This lead in the 1980s and 1990s to a series of studies that (re-) discovered adult neurogenesis in a variety of species including rat, mouse, rabbit, macaque and human and showed that newborn functional neurons can integrate into preexisting neuronal networks (Doetsch et al. 1997, 1999a; Eriksson et al. 1998; Gould et al. 1999a; Gueneau et al. 1982; Kempermann et al. 1997; Kornack and Rakic 1999; Kuhn et al. 1996; Stanfield and Trice 1988; van Praag et al. 2002). In addition, and equally important, systems started to be established to obtain cells from the adult brain that could generate neurons and glia in vitro (Lois and Alvarez-Buylla 1993; Reynolds and Weiss 1992).

These works, and several others, finally led to the acceptance of adult mammalian neurogenesis and to the following boost in neural stem cell research. It should be said, however, that this period of great discoveries was, and somehow still is, a period of confusion too. For example, while adult neurogenesis in the hippocampus and subventricular zone is firmly established, adult neurogenesis in the cortex has been *proven* (Dayer et al. 2005; Gould et al. 1999b, 2001) and *disproven* (Ackman et al. 2006; Frielingsdorf et al. 2004; Koketsu et al. 2003; Kornack and Rakic 2001) various times and certain observations on adult neurogenesis in humans (Curtis et al. 2007) have been openly challenged (Sanai et al. 2007). These conflicting reports, in part explained by an inappropriate use of recent technologies (Breunig et al. 2007a), reflect the novelty and dynamism of the field and should be kept in mind while studying adult neurogenesis.

Nevertheless, it took science nearly a century to change the "*harsh decree*" and view the adult brain as a dynamic and plastic organ where newborn neurons are integrated into existing circuits each day (Gross 2000). Adapting Cajal's statement one century later, we may now conclude that *it is for the science of the future to manipulate*, *if possible*, *brain plasticity for therapeutic intervention*.

12.4.2 Derivation/Classification

12.4.2.1 Origin

At the end of mammalian embryonic development, radial glial cells are believed to undergo a series of morphological and molecular changes that progressively transform them into astrocytes of the adult brain (Barry and McDermott 2005; Kriegstein and Alvarez-Buylla 2009; Mission et al. 1991; Voigt 1989). In particular, bipolar radial glia cells were recently observed by time-lapse microscopy on embryonic brain cultures to lose apical contact, migrate to the cortical plate and assume a stellate morphology characteristic of mature astrocytes (Noctor et al. 2008). This process is accompanied at neonatal stages by a reduction in the proliferative potential of newborn astrocytes as they progressively slow their rate of division to become quiescent (Ichikawa et al. 1983). Alternatively, some radial glial cells of specific brain regions escape this fate and, while keeping astrocytic features, become adult neural stem cells (Kriegstein and Alvarez-Buylla 2009).

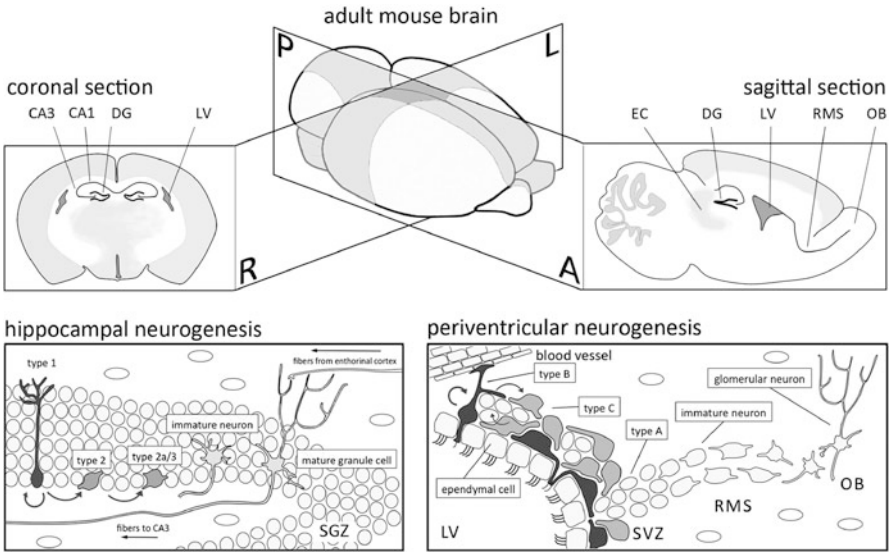


Fig. 12.3 The adult neurogenic niche. (*top*) Coronal (*left*) and sagittal (*right*) sections of the adult mouse brain (*center*; P-A-L-R = posterior-anterior-left-right, respectively) showing the sites of adult neurogeneis. (*bottom*) Cytoarchitecture of the SGZ (*left*) and SVZ (*right*). Type 1/B, 2/C, 3/A cells and neurons are depicted. *Arrows* indicate their lineage. *DG* dentate gyrus, *LV* lateral ventricle, *EC* entorhinal cortex, *RMS* rostral migratory stream, *OB* olfactory bulb, *SGZ* subgranular zone

Unfortunately, the terminology in the field may lead to confusion as radial glial cells of the developing CNS are neural stem cells but it would be inappropriate to call adult neural stem cells radial glia. Moreover, while adult neural stem cells have astrocytic features, not all astrocytes are neural stem cells. Nevertheless, the two regions of the adult brain in which neural stem cells have been more consistently reported and rigorously studied are the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle (Fig. 12.3) (Doetsch et al. 1997, 1999a; Eriksson et al. 1998; Gould et al. 1999a; Gueneau et al. 1982; Kempermann et al. 1997; Kornack and Rakic 1999; Kuhn et al. 1996; Stanfield and Trice 1988; van Praag et al. 2002). In these two neurogenic niches different stem and progenitor cells coexist that are reminiscent of embryonic precursors, as we shall see later.

12.4.2.2 Identification and Nomenclature

The terminology used to identify the various precursors of the SGZ differs from that of the SVZ because the two neurogenic niches have been independently characterized by different groups. This may seem unfortunate because neural stem and progenitor cells in these two areas are similar with regard to their origin, morphology

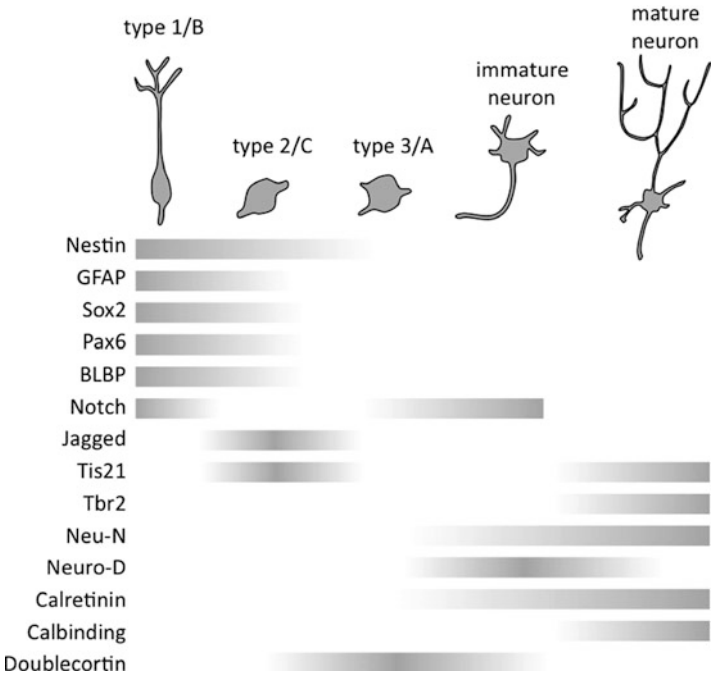


Fig. 12.4 Molecular markers commonly used to identify neural precursors in adult neurogenesis (Adapted from Attardo et al. (2008), Breunig et al. (2007b) and Zhao et al. (2008))

and function, which would call for a consistent terminology. However, important differences exist, in particular with regard to the expression of certain molecular markers and the neuronal subtypes generated, making it convenient to keep a different nomenclature for a more rigorous classification.

The main approach currently used to identify precursor subtypes in vivo is by performing immunohistochemistry with antibodies against specific molecular markers (Fig. 12.4). In essence, the detection of immunolabeling, or lack thereof, is taken as an evidence of cell identity. Though technically very practicable, the problematics inherent in such an approach are several such as that (1) no single individual marker has yet been described to selectively label an entire precursor subpopulation, (2) certain cell types are identified based on quantitative assessment of labeling intensity, which is often problematic, (3) immunolabeling does not necessarily indicate gene expression as synthesized proteins may be inherited from mother to daughter cell, and lastly, (4) cells may change expression levels of certain genes while progressing through the cell cycle and, thus, a different expression level of a marker may not necessarily indicate different cell identity. Due to these limitations, immunolabeling for molecular markers, or the use of transgenic reporter mouse lines for that particular marker, is typically combined with BrdU labeling and estimation of cell cycle parameters, which provides further evidence of cell identity because of the different cell cycle kinetics characteristic of precursor subtypes.

Finally, but certainly more difficult, cell identity could be ascertained by electron microscopy and ultrastructural analyses, which, together with advanced light microscopy, has been critical to 3D-reconstruct the cytoarchitecture of the neurogenic niches (Mirzadeh et al. 2008; Shen et al. 2008; Tavazoie et al. 2008). The combination of these techniques has led to the identification of three main precursors types in the SGZ and SVZ.

In the SGZ, neural precursors have been defined to as type 1, 2 and 3 with an additional subdivision of type 2 cells in 2a and 2b (Kempermann et al. 2004). Similarly, in the SVZ neural precursors have been defined to as type B, C and A cells, with the subdivision of B cells in type B1 and B2 as original proposed (Doetsch et al. 1997) being later abandoned (Doetsch et al. 1999a). The morphological and functional features of these precursor subtypes and their lineage will be more thoroughly described later; it suffices here to say that the type 1/B cells are thought to generate type 2/C cells, and these then generate type 3/A cells, also referred to as neuroblasts of newborn neurons.

Finally, a fourth cell type is present specifically in the periventricular area. These are ependymal cells forming a single-cell layer that delimits the boundary between the SVZ (also called subependymal zone) and the lumen of the ventricle. Ependymal cells were also proposed to be neural stem cells (Johansson et al. 1999) but this view was later disputed (Capela and Temple 2002; Chiasson et al. 1999; Doetsch et al. 1999a) leading to a long controversy in the field (Chojnacki et al. 2009). Recently, the neurogenic capacity of ependymal cells was shown to occur only during certain neurological disorders (Carlen et al. 2009) and, thus, the consensus at the time of writing is that ependymal cells are not neural stem cells under physiological conditions.

12.4.3 Characteristics/Properties

12.4.3.1 Anatomy and Cytoarchitecture of the Neurogenic Niches

The hippocampus lies within the temporal lobes of the telencephalic hemispheres and is more generally subdivided into CA1, CA3 and dentate gyrus, which form the trisynaptic circuit of this brain area. A high nuclear density in the three regions makes the hippocampus easy to identify on cross-sections. Specifically, CA1 (dorsally) and CA3 (ventrally) fuse to form the characteristic shape of a C (with its concavity oriented medially) while the dentate gyrus, resembling a V (with its concavity oriented laterally), is adjacent to CA3 (Fig. 12.3) (see the Allan Brain Atlas for 3D-reconstructions of brain anatomy: http://www.brain-map.org).

Simplifying a complex neuronal circuit, inputs to the hippocampus are transmitted from the entorhinal cortex to the dentate gyrus, which is connected to CA3 through the mossy fibers. Signals from pyramidal neurons in CA3 are then sent via the Schaffer collateral to CA1, which finally project outside the hippocampus back to the entorhinal cortex.

The only region of the hippocampus where neural stem cells are known to reside is the SGZ of the dentate gyrus, a particularly vascularized region with a diverse

population of highly packed cells, including type 1, 2 and 3 precursors. Advanced light microscopy has shown that bipolar, type 1 cells are oriented perpendicularly to the SGZ, have their nuclei towards the concavity of the V and span with their process the entire thickness of the SGZ until branching into several smaller processes at the level of the inner molecular layer, where they contact blood vessels. In contrast, type 2 and 3 cells lose radial morphology and assume a more rounded shape. Newborn neurons remain in the SGZ and extend axon and dendrites to integrate into the circuitry of the hippocampus (Fig. 12.3) (Alvarez-Buylla and Lim 2004; Kempermann et al. 2004; Kriegstein and Alvarez-Buylla 2009).

The anatomy and cytoarchitecture of the SVZ is very different from the hippocampus. With the end of embryonic development, the cavity of the neural tube collapses and is reduced to minimal proportions. Delimiting and in direct contact with the cerebrospinal fluid lies a single cell layer of ciliated ependymal cells, supposedly derived from radial glial cells (Kriegstein and Alvarez-Buylla 2009). In contrast to the hippocampus, which is both a neuronal network in itself and a neurogenic niche, the SVZ serves only the latter function. In fact, neurons derived from the SVZ migrate through the rostral migratory stream toward the rostral-most region of the brain, the olfactory bulbs, where they integrate into the networks mediating olfaction.

The olfactory bulbs receive information from the adjacent olfactory epithelium, laying the nasal cavity. In this context, it should be mentioned that the olfactory epithelium is yet another site of adult neurogenesis, though in this case neurons are part of the peripheral, rather than central, nervous system (Martinez-Marcos et al. 2000), which makes them somehow less attractive for therapy.

Advanced light microscopy and 3D-reconstruction by electron microscopy allowed to define the cytoarchitecture of the adult SVZ (Mirzadeh et al. 2008; Shen et al. 2008; Tavazoie et al. 2008). Relatively flat ependymal cells surround type B cells in a pin-wheel arrangement, which allows B cells to extend their primary cilium into the lumenal fluid. Nuclei of B cells are located underneath ependymal cells (Mirzadeh et al. 2008) and extend a basal process to surround C cells, which lack lumenal contact and themselves surround A cells. Finally, in the center of this B-C-A layering, type A cells form chains of migrating neuroblasts/newborn neurons forming the rostral migratory stream connecting the neurogenic niche with the final destination of the newborn neurons, the olfactory bulb.

12.4.3.2 Cell Biological Features of Adult Neural Stem Cells

It is interesting to observe that, despite many differences, adult neural stem and progenitor cells retain certain features of their functionally equivalent embryonic counterparts, i.e. radial glial cells and basal progenitors, respectively.

For example, adult type 1/B cells, like radial glia, are bipolar and highly elongated, and are characterized by the expression of astrocytic markers and the contact of their terminal process with blood vessels (Filippov et al. 2003; Fukuda et al. 2003; Shen et al. 2008; Tavazoie et al. 2008). In addition, they retain a primary cilium, an organelle long neglected through which important signaling pathways act (Doetsch et al. 1999b;

Mirzadeh et al. 2008). In contrast to type 1/B cells, and similar to basal progenitors, type 2/C progenitors assume a rounded morphology and, in the SVZ, lose contact with the lumen of the ventricle (Doetsch et al. 1999b; Mirzadeh et al. 2008). An important feature distinguishing adult neural stem cells from radial glia, besides the lack of interkinetic nuclear migration, concerns their cell cycle kinetics.

As already described, the developing cortex is a highly proliferative tissue with a cell cycle length of 10–20 h (Calegari et al. 2005; Takahashi et al. 1995), which is similar to type 2/C progenitors (Cameron and McKay 2001; Hayes and Nowakowski 2002; Morshead and van der Kooy 1992; Zhang et al. 2006). In contrast, the rate of division of type 1/B cells is extremely slow, which has earned them the epithet of slowly-dividing or label-retaining cells as S-phase tracers are less frequently diluted by cell division and, thus, can be detected in daughter stem cells a long time after incorporation into their mothers. The slow division rate of type 1/B cells has been demonstrated after administration of cytostatic agents that kill cells undergoing mitosis (in particular, arabinosyde-C (AraC))(Doetsch et al. 1999b; Morshead et al. 1994; Seri et al. 2001). First, AraC treatment depletes type 2/C and 3/A cells, but not type 1/B cells. Second, removal of AraC allows the repopulation of all cell types. Thus, type 1/B cells are mostly quiescent and are true stem cells as they alone can regenerate the entire neurogenic niche (Doetsch et al. 1999b; Morshead et al. 1994; Seri et al. 2001). Calculation upon different times of AraC treatment allowed to estimate cell cycle length of adult type 1/B neural stem cells that divide, on average every 2–4 weeks (Doetsch et al. 1999b; Morshead et al. 1994). This, however, should not be interpreted as to indicate that the cell cycle of neural stem cells requires several weeks to be completed; rather it suggests quiescence for most of this time, with the cell cycle length proper being a small fraction of it.

Finally, type 3/A cells, the third precursor type with no counterpart in the developing cortex, are also considered proliferating precursors, hence their name neuroblasts (Doetsch et al. 1999a), which implies that they undergo cell division. In fact, AraC treatment also depletes type 3/A cells, and a relatively short BrdU exposure is sufficient to label a proportion of them. The view that type 3/A cells progress through the cell cycle is supported by the expression, in a subpopulation of them, of markers of proliferation (Doetsch et al. 1999a). However, considering (1) the limitations in assessing cell identity by immunohistochemistry, (2) that depletion of type 2/C cells would alone be sufficient to deplete also their progeny, and (3) that BrdU is inherited from a proliferating mother to a postmitotic daughter, these experiments cannot exclude the possibility that a proportion of type 3/A cells are postmitotic neurons rather than proliferating precursors.

12.4.4 Differentiation Capacity and Their Precursors

12.4.4.1 Lineage and Mode of Division

As already mentioned, the exact lineage and mode of division of adult neural precursors is much less defined than those during embryonic development, which is

due to intrinsic difficulties in performing lineage tracing experiments in the adult brain. For example, contrary to the developing cortex, adult neural stem and progenitor cells constitute a particularly small population distributed within postmitotic neurons and glia. In addition, no individual marker for specific progenitor subtypes has yet been found and, thus, transgenic reporter mouse lines cannot be effectively used to identify the relevant cell type to image, which is very important for lineage tracing experiments in tissue. Finally, neural stem cells are mostly quiescent requiring very long culture experiments, which may be unphysiological or harmful to the tissue. Nevertheless, various morphological, functional and molecular studies suggest that type 1/B cells generate type 2/C cells while the latter generate type 3/A cells. No direct evidence is available to suggest that any given precursor undergoes symmetric or asymmetric cell division.

12.4.4.2 Factors Influencing Differentiation

For many decades, morphogens, transcription and trophic factors that influence neural differentiation have been almost exclusively studied during embryonic development. Not surprisingly, however, the same factors are now shown to have similar effects on adult neural stem cells. We will here only briefly summarize the most important examples of these signaling pathways and refer the reader to comprehensive reviews for more information (Ever and Gaiano 2005; Guillemot 2007; Ninkovic and Gotz 2007; Suh et al. 2009).

The vascular niche: Adult neurogenesis occurs is a highly vascularized environment, which suggests that signals may be transmitted from the blood to neural precursors in order to control their activity. The concept of a vascular niche (Palmer et al. 2000) (see also Chap. 5) was somehow present three decades ago from studies on seasonal songbirds as, in fact, adult neurogenesis was known to be triggered by hormones released into the blood (Nottebohm 1985). More recently, a direct link between testosterone, angiogenesis and adult neurogenesis has been established in birds (Louissaint et al. 2002) while neural precursors in mammals have been found to cluster around blood vessels (Shen et al. 2008; Tavazoie et al. 2008). The cross-talk between angiogenesis and neurogenesis is attracting now more attention (Alvarez-Buylla and Lim 2004; Suh et al. 2009), and it is presumably not a coincidence that factors promoting angiogenesis, most notably VEGF, also promote neurogenesis (Cao et al. 2004; Jin et al. 2002) while their inhibition has the opposite effect (Cao et al. 2004; Fabel et al. 2003). Finally, it is worth noting that increased angiogenesis is the primary response in many models of neurodegenerative disease and that treatments that improve angiogenesis also improve brain recovery after injury (Zhang and Chopp 2009).

Notch: Both Notch and its ligands Jagged1 and Dll1 are expressed in the adult neurogenic niche (Givogri et al. 2006; Nyfeler et al. 2005), and activation of the Notch pathway in adult neural stem cells promotes their expansion and inhibits differentiation (Androutsellis-Theotokis et al. 2006; Nyfeler et al. 2005). Interestingly, Notch activity, and thus neural precursor self-renewal, seems to be mediated by an autoregulatory loop between Notch and Shh signaling (Androutsellis-Theotokis et al. 2006),

which is also implicated in controlling quiescence of ependymal cells (Carlen et al. 2009). In addition, certain neurodegenerative diseases, such as stroke, induce Notch signaling (Carlen et al. 2009), and administration of Notch-ligands can be used to better restore brain function upon stroke (Androutsellis-Theotokis et al. 2006).

Shh: Similar to the effects observed during corticogenesis, activation of Shh signaling in adult neural precursors induces a strong, dose-dependent increase in proliferation while its inhibition has the opposite effect (Breunig et al. 2008; Lai et al. 2003; Machold et al. 2003). Interestingly, key mediators of the Shh pathway in adult neural stem cells are localized on the primary cilium (Breunig et al. 2008; Han et al. 2008), an organelle recognized to play important functions during development (Gerdes et al. 2009). Finally, transgenic mouse lines to genetically monitor Shh activity have allowed to fate-map neural precursors, providing the first evidence that quiescent neural stem cells can self-renew for over 1 year (Ahn and Joyner 2005).

Wnt: Again similar to the effects during corticogenesis, increasing Wnt signaling in the adult hippocampus promotes cell cycle progression and increases neuronal output while, conversely, inhibiting Wnt has the opposite effects (Lie et al. 2005). Wnt function in adult neural stem cells has recently been proposed to act through a sophisticated crosstalk between the transcription factor Sox2 and the proneural gene NeuroD1 (Kuwabara et al. 2009).

12.4.5 Function and Potential Application for Therapies

12.4.5.1 Role of Adult Neurogenesis

One of the most challenging goals in neuroscience is to understand the molecular and cellular mechanisms underlying elaborate cognitive functions. It is a fact, however, that this is one of the fields in life science where we still know relatively little. The physiological processes that allow learning, memory, feeling emotions and elaborate functions that are emphasized in humans, such as self-consciousness, are even difficult to conceptualize, but the recognition of adult neurogenesis has led scientists to investigate whether neural stem cells may be involved in any of these functions (Abrous et al. 2005; Imayoshi et al. 2009; Kempermann 2008; Lledo et al. 2006; Zhao et al. 2008).

A striking finding in this context is that external physiological stimuli have an effect on neurogenesis (Kempermann et al. 1997; Leuner et al. 2004). In the SGZ, genetic differences in various mouse strains have been correlated to the extent of precursors proliferation, neurogenesis and mouse performance in learning and memory tasks (Kempermann and Gage 2002). Moreover, voluntary exercise, such as allowing mice to train on a running wheel, or an enriched environment, such as larger housing conditions with toys and other mice for stimulating social behavior, increases neurogenesis and neuronal survival, respectively (Olson et al. 2006; Zhao et al. 2008). Interestingly, these stimuli also increase the mouse performance in learning and memory tasks (Olson et al. 2006; Zhao et al. 2008).

While these experiments could show a direct correlation between adult neurogenesis and learning and memory, other experiments aimed to manipulate adult neurogenesis failed to report consistent effects. For example, inhibiting adult hippocampal neurogenesis by depletion of precursors by means of X-ray irradiation did not prevent the beneficial effects on learning and memory that was induced by the subsequent exposure to an enriched environment. This suggests that factors other than neurogenesis, such as an increased angiogenesis or hormone activity, may be responsible for the improved behavioral response (Meshi et al. 2006).

In addition to radiation, cytostatic drugs or genetic manipulations are also used to investigate the behavioral effects upon inhibition of neurogenesis. Yet, alternative approaches have shown opposite outcomes (Olson et al. 2006; Saxe et al. 2006; Zhang et al. 2008a; Zhao et al. 2008). Thus, although adult hippocampal neurogenesis has been suggested to play a role in learning, memory, fear conditioning, depression and other elaborate brain functions, none of these effects are firmly established (Abrous et al. 2005; Imayoshi et al. 2009; Kempermann 2008; Lledo et al. 2006; Zhao et al. 2008).

The role of adult neurogenesis in olfaction is certainly less controversial. In this context, neurogenesis in the SVZ is essential to preserve cell homeostasis of the olfactory bulb as newborn neurons are needed to replace old, dying ones (Imayoshi et al. 2008). This is in contrast to the SGZ in which addition of newborn neurons is cumulative, leading to an increase, though extremely modest, in neuron number over time (Imayoshi et al. 2008). It is important to mention, however, that in both cases the vast majority of newborn neurons will not integrate into preexisting circuits but will undergo cell death. Nevertheless, the fact that neurogenesis is needed to preserve cell number in the olfactory bulb has corroborated the previous hypothesis that SVZ neurogenesis is essential for acquisition and memory of olfactory stimuli, which in rodents is of paramount importance for interacting with the environment and for social behavior (Alonso et al. 2006; Lledo et al. 2006).

12.4.5.2 Use of Neural Stem Cells in Therapy

A justification for the great efforts and huge investments in neural stem cell research is its possible application in therapies for neurodegenerative diseases or CNS injuries (Okano et al. 2007; Steiner et al. 2006; Zhang and Chopp 2009; Zuccato and Cattaneo 2009). As we shall see in this book, however, cell-based treatments of neural pathologies are at the moment the least developed and effective when compared to similar treatments in other tissues, such as in the bone marrow, skin, pancreas or bone (part IV of this book).

This gap is easily explained by the many difficulties inherent in the study and manipulation of the CNS in human patients and by the limited knowledge, relative to other organs, of its physiology. Moreover, adult neurogenesis in rodents, the main animal model for experimental research, greatly differs from neurogenesis in humans, and very few neural pathologies are faithfully reproduced in the laboratory, which makes it even harder to test novel strategies for therapy. Nevertheless, the

price at stake for developing effective therapies for neural disorders, a main cause of disease in rich countries, is great and first attempts in this direction have already been undertaken. In most cases, however, their efficacy is difficult to evaluate for it is unfeasible to "use" patients as negative control.

Admittedly, even if based on circumstantial evidence and a relatively scarce knowledge of human CNS physiology and pathology, the first attempts in this direction are promising as they, in some cases, have lead to major improvement of clinical conditions (see also Chaps. 29, 30, and 31). For example, transplantation of human embryonic brain tissue into patients with Parkinson Disease allowed one of six patients to suspend medical treatment for more than a year (Wenning et al. 1997). Others works, though also in principle successful, have instead raised more controversy. For example, neural cells have been cultured for several passages in vitro and then transplanted into brains of patients with stroke (Kondziolka et al. 2000). More recently, a similar approach has been used to treat patients with open brain injury (Zhu et al. 2006). The fact that cells passaged for several weeks, if not months, in culture may undergo transformation and lead to cancer after transplantation makes these attempts hazardous, if not ethically questionable.

Thanks to the revolutionary advent of induced pluripotency (Takahashi and Yamanaka 2006) we can now envision safer and more efficient systems to generate *patient-customized* stem cells for therapy (see also Chap. 8). Clearly, more work needs to be done to be able to control their differentiation into the desired cell type and to explore their use in therapy. This is the reason why experiments on animal models of neural disorders, including Alzheimer (Brinton and Wang 2006), Parkinson (Arias-Carrion and Yuan 2009), stroke (Zhang et al. 2008b) and spinal cord lesions (Okano and Sawamoto 2008) are so important.

Finally, it should be mentioned that certain brain tumors have now been shown to originate from an altered proliferation of neural precursors (Alcantara Llaguno et al. 2009; Wang et al. 2009) and, thus, studying neural stem cells may open up possibilities for understanding cancer and, perhaps, design new therapies for it (Colleoni and Torrente 2008).

12.4.6 Conclusions and Future Development in Research

It should be evident from this chapter that understanding the mechanisms underlying adult neurogenesis and its physiological function and manipulating this process for therapeutic purposes are all in a very preliminary phase. However, the momentum created by recent breakthroughs in the field and the great hopes that somatic stem cells carry for future therapies largely justify the huge investments devoted to it. While it is too early to predict the real usefulness of neural stem cell in therapy, there is little doubt that future basic research in this field will be crucial to better understand brain development and evolution.

References

Abrous DN, Koehl M, Le Moal M (2005) Adult neurogenesis: from precursors to network and physiology. Physiol Rev 85:523–569

Ackman JB, Siddiqi F, Walikonis RS, LoTurco JJ (2006) Fusion of microglia with pyramidal neurons after retroviral infection. J Neurosci 26:11413–11422

Ahn S, Joyner AL (2005) In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature 437:894–897

Aigner S, Denli AM, Gage FH (2007) A novel model for an older remodeler: the BAF swap in neurogenesis. Neuron 55:171–173

Alcantara Llaguno S, Chen J, Kwon CH, Jackson EL, Li Y, Burns DK, Alvarez-Buylla A, Parada LF (2009) Malignant astrocytomas originate from neural stem/progenitor cells in a somatic tumor suppressor mouse model. Cancer Cell 15:45–56

Alonso M, Viollet C, Gabellec MM, Meas-Yedid V, Olivo-Marin JC, Lledo PM (2006) Olfactory discrimination learning increases the survival of adult-born neurons in the olfactory bulb. J Neurosci 26:10508–10513

Altman J (1962) Are new neurons formed in the brains of adult mammals? Science 135:1127–1128

Alvarez-Buylla A, Lim DA (2004) For the long run: maintaining germinal niches in the adult brain. Neuron 41:683–686

Ambros V (2004) The functions of animal microRNAs. Nature 431:350–355

Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD (2006) Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 442:823–826

Arai Y, Funatsu N, Numayama-Tsuruta K, Nomura T, Nakamura S, Osumi N (2005) Role of Fabp7, a downstream gene of Pax6, in the maintenance of neuroepithelial cells during early embryonic development of the rat cortex. J Neurosci 25:9752–9761

Arias-Carrion O, Yuan TF (2009) Autologous neural stem cell transplantation: a new treatment option for Parkinson's disease? Med Hypotheses 73(5):757–759

Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science 284:770–776

Attardo A, Calegari F, Haubensak W, Wilsch-Bräuninger M, Huttner WB (2008) Live imaging at the onset of cortical neurogenesis reveals differential appearance of the neuronal phenotype in apical versus basal progenitor progeny. PLoS One 3:e2388

Barry D, McDermott K (2005) Differentiation of radial glia from radial precursor cells and transformation into astrocytes in the developing rat spinal cord. Glia 50:187–197

Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136:215–233

Bautch VL, James JM (2009) Neurovascular development: the beginning of a beautiful friendship. Cell Adh Migr 3:199–204

Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ (2003) Dicer is essential for mouse development. Nat Genet 35:215–217

Biron VL, McManus KJ, Hu N, Hendzel MJ, Underhill DA (2004) Distinct dynamics and distribution of histone methyl-lysine derivatives in mouse development. Dev Biol 276:337–351

Borello U, Cobos I, Long JE, McWhirter JR, Murre C, Rubenstein JL (2008) FGF15 promotes neurogenesis and opposes FGF8 function during neocortical development. Neural Dev 3:17

BoulderComm (1970) Embryonic vertebrate central nervous system: revised terminology. The Boulder Committee. Anat Rec 166:257–261

Breunig JJ, Arellano JI, Macklis JD, Rakic P (2007a) Everything that glitters isn't gold: a critical review of postnatal neural precursor analyses. Cell Stem Cell 1:612–627

Breunig JJ, Silbereis J, Vaccarino FM, Sestan N, Rakic P (2007b) Notch regulates cell fate and dendrite morphology of newborn neurons in the postnatal dentate gyrus. Proc Natl Acad Sci USA 104:20558–20563

Breunig JJ, Sarkisian MR, Arellano JI, Morozov YM, Ayoub AE, Sojitra S, Wang B, Flavell RA, Rakic P, Town T (2008) Primary cilia regulate hippocampal neurogenesis by mediating sonic hedgehog signaling. Proc Natl Acad Sci USA 105:13127–13132

Brinton RD, Wang JM (2006) Therapeutic potential of neurogenesis for prevention and recovery from Alzheimer's disease: allopregnanolone as a proof of concept neurogenic agent. Curr Alzheimer Res 3:185–190

Briscoe J, Pierani A, Jessell TM, Ericson J (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101:435–445

Calegari F, Haubensak W, Haffner C, Huttner WB (2005) Selective lengthening of the cell cycle in the neurogenic subpopulation of neural progenitor cells during mouse brain development. J Neurosci 25:6533–6538

Cameron HA, McKay RD (2001) Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol 435:406–417

Cao L, Jiao X, Zuzga DS, Liu Y, Fong DM, Young D, During MJ (2004) VEGF links hippocampal activity with neurogenesis, learning and memory. Nat Genet 36:827–835

Cao X, Yeo G, Muotri AR, Kuwabara T, Gage FH (2006) Noncoding RNAs in the mammalian central nervous system. Annu Rev Neurosci 29:77–103

Cao X, Pfaff SL, Gage FH (2007) A functional study of miR-124 in the developing neural tube. Genes Dev 21:531–536

Capela A, Temple S (2002) LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. Neuron 35:865–875

Carlen M, Meletis K, Goritz C, Darsalia V, Evergren E, Tanigaki K, Amendola M, Barnabe-Heider F, Yeung MS, Naldini L, Honjo T, Kokaia Z, Shupliakov O, Cassidy RM, Lindvall O, Frisen J (2009) Forebrain ependymal cells are Notch-dependent and generate neuroblasts and astrocytes after stroke. Nat Neurosci 12:259–267

Castro DS, Skowronska-Krawczyk D, Armant O, Donaldson IJ, Parras C, Hunt C, Critchley JA, Nguyen L, Gossler A, Göttgens B, Matter JM, Guillemot F (2006) Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Dev Cell 11:831–844

Caviness VS, Takahashi T, Nowakowski RS (1995) Numbers, time and neocortical neuronogenesis: a general developmental and evolutionary model. Trends Neurosci 18:379–383

Cayuso J, Martí E (2005) Morphogens in motion: growth control of the neural tube. J Neurobiol 64:376–387

Cayuso J, Ulloa F, Cox B, Briscoe J, Martí E (2006) The Sonic hedgehog pathway independently controls the patterning, proliferation and survival of neuroepithelial cells by regulating Gli activity. Development 133:517–528

Cheng LC, Tavazoie M, Doetsch F (2005) Stem cells: from epigenetics to microRNAs. Neuron 46:363–367

Cheng LC, Pastrana E, Tavazoie M, Doetsch F (2009) miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. Nat Neurosci 12:399–408

Chenn A, McConnell SK (1995) Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. Cell 82:631–641

Chenn A, Walsh CA (2003) Increased neuronal production, enlarged forebrains and cytoarchitectural distortions in beta-catenin overexpressing transgenic mice. Cereb Cortex 13:599–606

Chesnutt C, Burrus LW, Brown AM, Niswander L (2004) Coordinate regulation of neural tube patterning and proliferation by TGFbeta and WNT activity. Dev Biol 274:334–347

Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA (1996) Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383:407–413

Chiasson BJ, Tropepe V, Morshead CM, van der Kooy D (1999) Adult mammalian forebrain ependymal and subependymal cells demonstrate proliferative potential, but only subependymal cells have neural stem cell characteristics. J Neurosci 19:4462–4471

Chojnacki AK, Mak GK, Weiss S (2009) Identity crisis for adult periventricular neural stem cells: subventricular zone astrocytes, ependymal cells or both? Nat Rev Neurosci 10:153–163

Colleoni F, Torrente Y (2008) The new challenge of stem cell: brain tumour therapy. Cancer Lett 272:1–11

Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelso C, Holtas S, van Roon-Mom WM, Bjork-Eriksson T, Nordborg C, Frisen J, Dragunow M, Faull RL, Eriksson PS (2007) Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. Science 315:1243–1249

Dahmane N, Sánchez P, Gitton Y, Palma V, Sun T, Beyna M, Weiner H, Ruiz i Altaba A (2001) The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. Development 128:5201–5212

Dayer AG, Cleaver KM, Abouantoun T, Cameron HA (2005) New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol 168:415–427

De Pietri Tonelli D, Pulvers JN, Haffner C, Murchison EP, Hannon GJ, Huttner WB (2008) miRNAs are essential for survival and differentiation of newborn neurons but not for expansion of neural progenitors during early neurogenesis in the mouse embryonic neocortex. Development 135:3911–3921

Dehay C, Kennedy H (2007) Cell-cycle control and cortical development. Nat Rev Neurosci 8:438–450

Dickinson ME, Krumlauf R, McMahon AP (1994) Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system. Development 120:1453–1471

Diez del Corral R, Breitkreuz DN, Storey KG (2002) Onset of neuronal differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling. Development 129:1681–1691

Diez del Corral R, Olivera-Martinez I, Goriely A, Gale E, Maden M, Storey K (2003) Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron 40:65–79

Doe CQ, Skeath JB (1996) Neurogenesis in the insect central nervous system. Curr Opin Neurobiol 6:18–24

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A (1997) Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J Neurosci 17:5046–5061

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999a) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A (1999b) Regeneration of a germinal layer in the adult mammalian brain. Proc Natl Acad Sci USA 96:11619–11624

Du T, Zamore PD (2005) microPrimer: the biogenesis and function of microRNA. Development 132:4645–4652

Ekholm SV, Reed SI (2000) Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle. Curr Opin Cell Biol 12:676–684

Erecińska M, Silver IA (2001) Tissue oxygen tension and brain sensitivity to hypoxia. Respir Physiol 128:263–276

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313–1317

Estivill-Torrus G, Pearson H, van Heyningen V, Price DJ, Rashbass P (2002) Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors. Development 129:455–466

Ever L, Gaiano N (2005) Radial 'glial' progenitors: neurogenesis and signaling. Curr Opin Neurobiol 15:29–33

Fabel K, Tam B, Kaufer D, Baiker A, Simmons N, Kuo CJ, Palmer TD (2003) VEGF is necessary for exercise-induced adult hippocampal neurogenesis. Eur J Neurosci 18:2803–2812

Fan G, Martinowich K, Chin MH, He F, Fouse SD, Hutnick L, Hattori D, Ge W, Shen Y, Wu H, ten Hoeve J, Shuai K, Sun YE (2005) DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. Development 132:3345–3356

Farkas LM, Huttner WB (2008) The cell biology of neural stem and progenitor cells and its significance for their proliferation versus differentiation during mammalian brain development. Curr Opin Cell Biol 20:707–715

Filippov V, Kronenberg G, Pivneva T, Reuter K, Steiner B, Wang LP, Yamaguchi M, Kettenmann H, Kempermann G (2003) Subpopulation of nestin-expressing progenitor cells in the adult

murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. Mol Cell Neurosci 23:373–382
Firket H, Verly WG (1958) Autoradiographic visualization of synthesis of deoxyribonucleic acid in tissue culture with tritium-labelled thymidine. Nature 181:274–275
Fish JL, Kosodo Y, Enard W, Pääbo S, Huttner WB (2006) Aspm specifically maintains symmetric proliferative divisions of neuroepithelial cells. Proc Natl Acad Sci USA 103:10438–10443
Fish JL, Dehay C, Kennedy H, Huttner WB (2008) Making bigger brains-the evolution of neural-progenitor-cell division. J Cell Sci 121:2783–2793
Fode C, Ma Q, Casarosa S, Ang SL, Anderson DJ, Guillemot F (2000) A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. Genes Dev 14:67–80
Frielingsdorf H, Schwarz K, Brundin P, Mohapel P (2004) No evidence for new dopaminergic neurons in the adult mammalian substantia nigra. Proc Natl Acad Sci USA 101:10177–10182
Fukuda S, Kato F, Tozuka Y, Yamaguchi M, Miyamoto Y, Hisatsune T (2003) Two distinct subpopulations of nestin-positive cells in adult mouse dentate gyrus. J Neurosci 23:9357–9366
Gavin BJ, McMahon JA, McMahon AP (1990) Expression of multiple novel Wnt-1/int-1-related genes during fetal and adult mouse development. Genes Dev 4:2319–2332
Gerdes JM, Davis EE, Katsanis N (2009) The vertebrate primary cilium in development, homeostasis, and disease. Cell 137:32–45
Givogri MI, de Planell M, Galbiati F, Superchi D, Gritti A, Vescovi A, de Vellis J, Bongarzone ER (2006) Notch signaling in astrocytes and neuroblasts of the adult subventricular zone in health and after cortical injury. Dev Neurosci 28:81–91
Götz M, Huttner WB (2005) The cell biology of neurogenesis. Nat Rev Mol Cell Biol 6:777–788
Götz M, Stoykova A, Gruss P (1998) Pax6 controls radial glia differentiation in the cerebral cortex. Neuron 21:1031–1044
Gould E, Reeves AJ, Fallah M, Tanapat P, Gross CG, Fuchs E (1999a) Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci USA 96:5263–5267
Gould E, Reeves AJ, Graziano MS, Gross CG (1999b) Neurogenesis in the neocortex of adult primates. Science 286:548–552
Gould E, Vail N, Wagers M, Gross CG (2001) Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. Proc Natl Acad Sci USA 98:10910–10917
Gross CG (2000) Neurogenesis in the adult brain: death of a dogma. Nat Rev Neurosci 1:67–73
Gueneau G, Privat A, Drouet J, Court L (1982) Subgranular zone of the dentate gyrus of young rabbits as a secondary matrix. A high-resolution autoradiographic study. Dev Neurosci 5:345–358
Guillemot F (2007) Cell fate specification in the mammalian telencephalon. Prog Neurobiol 83:37–52
Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, Lundkvist J, Ruas JL, Poellinger L, Lendahl U, Bondesson M (2005) Hypoxia requires notch signaling to maintain the undifferentiated cell state. Dev Cell 9:617–628
Han YG, Spassky N, Romaguera-Ros M, Garcia-Verdugo JM, Aguilar A, Schneider-Maunoury S, Alvarez-Buylla A (2008) Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. Nat Neurosci 11:277–284
Hatakeyama J, Bessho Y, Katoh K, Ookawara S, Fujioka M, Guillemot F, Kageyama R (2004) Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development 131:5539–5550
Haubensak W, Attardo A, Denk W, Huttner WB (2004) Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. Proc Natl Acad Sci USA 101:3196–3201
Hayes NL, Nowakowski RS (2000) Exploiting the dynamics of S-phase tracers in developing brain: interkinetic nuclear migration for cells entering versus leaving the S-phase. Dev Neurosci 22:44–55
Hayes NL, Nowakowski RS (2002) Dynamics of cell proliferation in the adult dentate gyrus of two inbred strains of mice. Brain Res Dev Brain Res 134:77–85

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW (1998) Identification of c-MYC as a target of the APC pathway. Science 281:1509–1512
Hevner RF (2006) From radial glia to pyramidal-projection neuron: transcription factor cascades in cerebral cortex development. Mol Neurobiol 33:33–50
Honjo T (1996) The shortest path from the surface to the nucleus: RBP-J kappa/Su(H) transcription factor. Genes Cells 1:1–9
Houbaviy HB, Murray MF, Sharp PA (2003) Embryonic stem cell-specific MicroRNAs. Dev Cell 5:351–358
Hu G, Lee H, Price SM, Shen MM, Abate-Shen C (2001) Msx homeobox genes inhibit differentiation through upregulation of cyclin D1. Development 128:2373–2384
Huttner WB, Brand M (1997) Asymmetric division and polarity of neuroepithelial cells. Curr Opin Neurobiol 7:29–39
Ichikawa M, Shiga T, Hirata Y (1983) Spatial and temporal pattern of postnatal proliferation of glial cells in the parietal cortex of the rat. Brain Res 285:181–187
Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R (2008) Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat Neurosci 11:1153–1161
Imayoshi I, Sakamoto M, Ohtsuka T, Kageyama R (2009) Continuous neurogenesis in the adult brain. Dev Growth Differ 51:379–386
Ingham PW, McMahon AP (2001) Hedgehog signaling in animal development: paradigms and principles. Genes Dev 15:3059–3087
Ishibashi M, McMahon AP (2002) A sonic hedgehog-dependent signaling relay regulates growth of diencephalic and mesencephalic primordia in the early mouse embryo. Development 129:4807–4819
Jacob J, Briscoe J (2003) Gli proteins and the control of spinal-cord patterning. EMBO Rep 4:761–765
Jessell TM (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet 1:20–29
Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA (2002) Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc Natl Acad Sci USA 99:11946–11950
Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisen J (1999) Identification of a neural stem cell in the adult mammalian central nervous system. Cell 96:25–34
Kageyama R, Nakanishi S (1997) Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. Curr Opin Genet Dev 7:659–665
Kageyama R, Ohtsuka T, Kobayashi T (2008) Roles of Hes genes in neural development. Dev Growth Differ 50(Suppl 1):S97–S103
Kaplan MS, Hinds JW (1977) Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197:1092–1094
Kempermann G (2008) The neurogenic reserve hypothesis: what is adult hippocampal neurogenesis good for? Trends Neurosci 31:163–169
Kempermann G, Gage FH (2002) Genetic influence on phenotypic differentiation in adult hippocampal neurogenesis. Brain Res Dev Brain Res 134:1–12
Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493–495
Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. Trends Neurosci 27:447–452
Kenney AM, Rowitch DH (2000) Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. Mol Cell Biol 20:9055–9067
Kenney AM, Cole MD, Rowitch DH (2003) Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors. Development 130:15–28
Kim VN (2005) MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 6:376–385

Klein ME, Impey S, Goodman RH (2005) Role reversal: the regulation of neuronal gene expression by microRNAs. Curr Opin Neurobiol 15:507–513
Koketsu D, Mikami A, Miyamoto Y, Hisatsune T (2003) Nonrenewal of neurons in the cerebral neocortex of adult macaque monkeys. J Neurosci 23:937–942
Komada M, Saitsu H, Kinboshi M, Miura T, Shiota K, Ishibashi M (2008) Hedgehog signaling is involved in development of the neocortex. Development 135:2717–2727
Kondziolka D, Wechsler L, Goldstein S, Meltzer C, Thulborn KR, Gebel J, Jannetta P, DeCesare S, Elder EM, McGrogan M, Reitman MA, Bynum L (2000) Transplantation of cultured human neuronal cells for patients with stroke. Neurology 55:565–569
Konno D, Shioi G, Shitamukai A, Mori A, Kiyonari H, Miyata T, Matsuzaki F (2008) Neuroepithelial progenitors undergo LGN-dependent planar divisions to maintain self-renewability during mammalian neurogenesis. Nat Cell Biol 10:93–101
Korada S, Zheng W, Basilico C, Schwartz ML, Vaccarino FM (2002) Fibroblast growth factor 2 is necessary for the growth of glutamate projection neurons in the anterior neocortex. J Neurosci 22:863–875
Kornack DR, Rakic P (1999) Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proc Natl Acad Sci USA 96:5768–5773
Kornack DR, Rakic P (2001) Cell proliferation without neurogenesis in adult primate neocortex. Science 294:2127–2130
Kosodo Y, Röper K, Haubensak W, Marzesco AM, Corbeil D, Huttner WB (2004) Asymmetric distribution of the apical plasma membrane during neurogenic divisions of mammalian neuroepithelial cells. EMBO J 23:2314–2324
Kriegstein A, Alvarez-Buylla A (2009) The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32:149–184
Kriegstein AR, Götz M (2003) Radial glia diversity: a matter of cell fate. Glia 43:37–43
Kuhn HG, Dickinson-Anson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 16:2027–2033
Kuwabara T, Hsieh J, Muotri A, Yeo G, Warashina M, Lie DC, Moore L, Nakashima K, Asashima M, Gage FH (2009) Wnt-mediated activation of NeuroD1 and retro-elements during adult neurogenesis. Nat Neurosci 12(9):1097–1105
Lai K, Kaspar BK, Gage FH, Schaffer DV (2003) Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nat Neurosci 6:21–27
Lange C, Mix E, Rateitschak K, Rolfs A (2006) Wnt signal pathways and neural stem cell differentiation. Neurodegener Dis 3:76–86
Lange C, Huttner WB, Calegari F (2009) Cdk4/cyclinD1 overexpression in neural stem cells shortens G1, delays neurogenesis, and promotes the generation and expansion of basal progenitors. Cell Stem Cell 5:320–331
Lathia JD, Mattson MP, Cheng A (2008) Notch: from neural development to neurological disorders. J Neurochem 107:1471–1481
Lessard J, Wu JI, Ranish JA, Wan M, Winslow MM, Staahl BT, Wu H, Aebersold R, Graef IA, Crabtree GR (2007) An essential switch in subunit composition of a chromatin remodeling complex during neural development. Neuron 55:201–215
Leuner B, Mendolia-Loffredo S, Kozorovitskiy Y, Samburg D, Gould E, Shors TJ (2004) Learning enhances the survival of new neurons beyond the time when the hippocampus is required for memory. J Neurosci 24:7477–7481
Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69:915–926
Lie DC, Colamarino SA, Song HJ, Desire L, Mira H, Consiglio A, Lein ES, Jessberger S, Lansford H, Dearie AR, Gage FH (2005) Wnt signalling regulates adult hippocampal neurogenesis. Nature 437:1370–1375
Liem KF, Tremml G, Jessell TM (1997) A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. Cell 91:127–138
Liu Y, Helms AW, Johnson JE (2004) Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development 131:1017–1028

Lledo PM, Alonso M, Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci 7:179–193
Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20:781–810
Lois C, Alvarez-Buylla A (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci USA 90:2074–2077
Lok J, Gupta P, Guo S, Kim WJ, Whalen MJ, van Leyen K, Lo EH (2007) Cell-cell signaling in the neurovascular unit. Neurochem Res 32:2032–2045
Lopez-Munoz F, Boya J, Alamo C (2006) Neuron theory, the cornerstone of neuroscience, on the centenary of the Nobel Prize award to Santiago Ramon y Cajal. Brain Res Bull 70:391–405
Louissaint A Jr, Rao S, Leventhal C, Goldman SA (2002) Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron 34:945–960
Lukaszewicz A, Savatier P, Cortay V, Kennedy H, Dehay C (2002) Contrasting effects of basic fibroblast growth factor and neurotrophin 3 on cell cycle kinetics of mouse cortical stem cells. J Neurosci 22:6610–6622
Machold R, Hayashi S, Rutlin M, Muzumdar MD, Nery S, Corbin JG, Gritli-Linde A, Dellovade T, Porter JA, Rubin LL, Dudek H, McMahon AP, Fishell G (2003) Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron 39:937–950
Machon O, van den Bout CJ, Backman M, Kemler R, Krauss S (2003) Role of beta-catenin in the developing cortical and hippocampal neuroepithelium. Neuroscience 122:129–143
Martí E, Takada R, Bumcrot DA, Sasaki H, McMahon AP (1995) Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. Development 121:2537–2547
Martinez-Marcos A, Ubeda-Banon I, Deng L, Halpern M (2000) Neurogenesis in the vomeronasal epithelium of adult rats: evidence for different mechanisms for growth and neuronal turnover. J Neurobiol 44:423–435
Martinowich K, Hattori D, Wu H, Fouse S, He F, Hu Y, Fan G, Sun YE (2003) DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. Science 302:890–893
Mason I (2007) Initiation to end point: the multiple roles of fibroblast growth factors in neural development. Nat Rev Neurosci 8:583–596
McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP (1998) Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev 12:1438–1452
Megason SG, McMahon AP (2002) A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. Development 129:2087–2098
Meshi D, Drew MR, Saxe M, Ansorge MS, David D, Santarelli L, Malapani C, Moore H, Hen R (2006) Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. Nat Neurosci 9:729–731
Mirzadeh Z, Merkle FT, Soriano-Navarro M, Garcia-Verdugo JM, Alvarez-Buylla A (2008) Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. Cell Stem Cell 3:265–278
Mission JP, Takahashi T, Caviness VS Jr (1991) Ontogeny of radial and other astroglial cells in murine cerebral cortex. Glia 4:138–148
Miyata T, Kawaguchi A, Saito K, Kawano M, Muto T, Ogawa M (2004) Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. Development 131:3133–3145
Morin X, Jaouen F, Durbec P (2007) Control of planar divisions by the G-protein regulator LGN maintains progenitors in the chick neuroepithelium. Nat Neurosci 10:1440–1448
Morshead CM, van der Kooy D (1992) Postmitotic death is the fate of constitutively proliferating cells in the subependymal layer of the adult mouse brain. J Neurosci 12:249–256
Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D (1994) Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron 13:1071–1082
Nakayama KI, Nakayama K (2006) Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer 6:369–381

Ninkovic J, Gotz M (2007) Signaling in adult neurogenesis: from stem cell niche to neuronal networks. Curr Opin Neurobiol 17:338–344
Noctor SC, Martínez-Cerdeño V, Ivic L, Kriegstein AR (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7:136–144
Noctor SC, Martínez-Cerdeño V, Kriegstein AR (2008) Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. J Comp Neurol 508:28–44
Nottebohm F (1985) Neuronal replacement in adulthood. Ann N Y Acad Sci 457:143–161
Nusse R, Fuerer C, Ching W, Harnish K, Logan C, Zeng A, ten Berge D, Kalani Y (2008) Wnt signaling and stem cell control. Cold Spring Harb Symp Quant Biol 73:59–66
Nyfeler Y, Kirch RD, Mantei N, Leone DP, Radtke F, Suter U, Taylor V (2005) Jagged1 signals in the postnatal subventricular zone are required for neural stem cell self-renewal. EMBO J 24:3504–3515
Ochiai W, Nakatani S, Takahara T, Kainuma M, Masaoka M, Minobe S, Namihira M, Nakashima K, Sakakibara A, Ogawa M, Miyata T (2009) Periventricular notch activation and asymmetric Ngn2 and Tbr2 expression in pair-generated neocortical daughter cells. Mol Cell Neurosci 40:225–233
Ohnuma S, Harris WA (2003) Neurogenesis and the cell cycle. Neuron 40:199–208
Ohnuma S, Philpott A, Harris WA (2001) Cell cycle and cell fate in the nervous system. Curr Opin Neurobiol 11:66–73
Ohtsuka T, Ishibashi M, Gradwohl G, Nakanishi S, Guillemot F, Kageyama R (1999) Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J 18:2196–2207
Okano H (2002) Stem cell biology of the central nervous system. J Neurosci Res 69:698–707
Okano H, Sawamoto K (2008) Neural stem cells: involvement in adult neurogenesis and CNS repair. Philos Trans R Soc Lond B Biol Sci 363:2111–2122
Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99:247–257
Okano H, Sakaguchi M, Ohki K, Suzuki N, Sawamoto K (2007) Regeneration of the central nervous system using endogenous repair mechanisms. J Neurochem 102:1459–1465
Olson AK, Eadie BD, Ernst C, Christie BR (2006) Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. Hippocampus 16:250–260
Osumi N, Shinohara H, Numayama-Tsuruta K, Maekawa M (2008) Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. Stem Cells 26:1663–1672
Palmer TD, Willhoite AR, Gage FH (2000) Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 425:479–494
Panchision DM (2009) The role of oxygen in regulating neural stem cells in development and disease. J Cell Physiol 220:562–568
Panchision DM, Pickel JM, Studer L, Lee SH, Turner PA, Hazel TG, McKay RD (2001) Sequential actions of BMP receptors control neural precursor cell production and fate. Genes Dev 15:2094–2110
Parr BA, Shea MJ, Vassileva G, McMahon AP (1993) Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. Development 119:247–261
Parras CM, Schuurmans C, Scardigli R, Kim J, Anderson DJ, Guillemot F (2002) Divergent functions of the proneural genes Mash1 and Ngn2 in the specification of neuronal subtype identity. Genes Dev 16:324–338
Perez SE, Rebelo S, Anderson DJ (1999) Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. Development 126:1715–1728
Pierani A, Brenner-Morton S, Chiang C, Jessell TM (1999) A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell 97:903–915
Pierani A, Moran-Rivard L, Sunshine MJ, Littman DR, Goulding M, Jessell TM (2001) Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. Neuron 29:367–384

Pilaz LJ, Patti D, Marcy G, Ollier E, Pfister S, Douglas RJ, Betizeau M, Gautier E, Cortay V, Doerflinger N, Kennedy H, Dehay C (2009) Forced G1-phase reduction alters mode of division, neuron number, and laminar phenotype in the cerebral cortex. Proc Natl Acad Sci USA 106(5):21924–21929

Pistollato F, Chen HL, Schwartz PH, Basso G, Panchision DM (2007) Oxygen tension controls the expansion of human CNS precursors and the generation of astrocytes and oligodendrocytes. Mol Cell Neurosci 35:424–435

Pollard SM, Wallbank R, Tomlinson S, Grotewold L, Smith A (2008) Fibroblast growth factor induces a neural stem cell phenotype in foetal forebrain progenitors and during embryonic stem cell differentiation. Mol Cell Neurosci 38:393–403

Qian X, Shen Q, Goderie SK, He W, Capela A, Davis AA, Temple S (2000) Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron 28:69–80

Raballo R, Rhee J, Lyn-Cook R, Leckman JF, Schwartz ML, Vaccarino FM (2000) Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. J Neurosci 20:5012–5023

Ramon y Cajal S (1913) Degeneracion y regeneracion de los niervos. Imprenta de hijos de Nicolas Moya, Madrid

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710

Reynolds BA, Weiss S (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175:1–13

Reynolds BA, Tetzlaff W, Weiss S (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12:4565–4574

Ringe J, Kaps C, Burmester GR, Sittinger M (2002) Stem cells for regenerative medicine: advances in the engineering of tissues and organs. Naturwissenschaften 89:338–351

Robertson KD, Wolffe AP (2000) DNA methylation in health and disease. Nat Rev Genet 1:11–19

Ross SE, Greenberg ME, Stiles CD (2003) Basic helix-loop-helix factors in cortical development. Neuron 39:13–25

Rottach A, Leonhardt H, Spada F (2009) DNA methylation-mediated epigenetic control. J Cell Biochem 108:43–51

Sanai N, Berger MS, Garcia-Verdugo JM, Alvarez-Buylla A (2007) Comment on "Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension". Science 318:393; author reply 393

Sansom SN, Griffiths DS, Faedo A, Kleinjan DJ, Ruan Y, Smith J, van Heyningen V, Rubenstein JL, Livesey FJ (2009) The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. PLoS Genet 5:e1000511

Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR (2006) Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. Proc Natl Acad Sci USA 103:17501–17506

Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21:7153–7160

Seroogy KB, Gall CM, Lee DC, Kornblum HI (1995) Proliferative zones of postnatal rat brain express epidermal growth factor receptor mRNA. Brain Res 670:157–164

Shastri VP (2006) Future of regenerative medicine: challenges and hurdles. Artif Organs 30:828–834

Shen Q, Wang Y, Kokovay E, Lin G, Chuang SM, Goderie SK, Roysam B, Temple S (2008) Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. Cell Stem Cell 3:289–300

Shi Y, Massagué J (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113:685–700

Shi Y, Sun G, Zhao C, Stewart R (2008) Neural stem cell self-renewal. Crit Rev Oncol Hematol 65:43–53

Shimogori T, Banuchi V, Ng HY, Strauss JB, Grove EA (2004) Embryonic signaling centers expressing BMP, WNT and FGF proteins interact to pattern the cerebral cortex. Development 131:5639–5647

Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A (1999) The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 96:5522–5527

Simpson TI, Price DJ (2002) Pax6; a pleiotropic player in development. Bioessays 24:1041–1051

Smirnova L, Gräfe A, Seiler A, Schumacher S, Nitsch R, Wulczyn FG (2005) Regulation of miRNA expression during neural cell specification. Eur J Neurosci 21:1469–1477

Stanfield BB, Trice JE (1988) Evidence that granule cells generated in the dentate gyrus of adult rats extend axonal projections. Exp Brain Res 72:399–406

Steiner B, Wolf S, Kempermann G (2006) Adult neurogenesis and neurodegenerative disease. Regen Med 1:15–28

Storm EE, Garel S, Borello U, Hebert JM, Martinez S, McConnell SK, Martin GR, Rubenstein JL (2006) Dose-dependent functions of Fgf8 in regulating telencephalic patterning centers. Development 133:1831–1844

Stoykova A, Fritsch R, Walther C, Gruss P (1996) Forebrain patterning defects in Small eye mutant mice. Development 122:3453–3465

Strahl BD, Allis CD (2000) The language of covalent histone modifications. Nature 403:41–45

Suh MR, Lee Y, Kim JY, Kim SK, Moon SH, Lee JY, Cha KY, Chung HM, Yoon HS, Moon SY, Kim VN, Kim KS (2004) Human embryonic stem cells express a unique set of microRNAs. Dev Biol 270:488–498

Suh H, Deng W, Gage FH (2009) Signaling in adult neurogenesis. Annu Rev Cell Dev Biol 25:253–275

Sun G, Yu RT, Evans RM, Shi Y (2007) Orphan nuclear receptor TLX recruits histone deacetylases to repress transcription and regulate neural stem cell proliferation. Proc Natl Acad Sci USA 104:15282–15287

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676

Takahashi T, Nowakowski RS, Caviness VS (1995) The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. J Neurosci 15:6046–6057

Takizawa T, Nakashima K, Namihira M, Ochiai W, Uemura A, Yanagisawa M, Fujita N, Nakao M, Taga T (2001) DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell 1:749–758

Tavazoie M, Van der Veken L, Silva-Vargas V, Louissaint M, Colonna L, Zaidi B, Garcia-Verdugo JM, Doetsch F (2008) A specialized vascular niche for adult neural stem cells. Cell Stem Cell 3:279–288

Tetsu O, McCormick F (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398:422–426

Toresson H, Potter SS, Campbell K (2000) Genetic control of dorsal-ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. Development 127:4361–4371

Turner BM (2002) Cellular memory and the histone code. Cell 111:285–291

Ulloa F, Briscoe J (2007) Morphogens and the control of cell proliferation and patterning in the spinal cord. Cell Cycle 6:2640–2649

Vaccarino FM, Schwartz ML, Raballo R, Nilsen J, Rhee J, Zhou M, Doetschman T, Coffin JD, Wyland JJ, Hung YT (1999a) Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. Nat Neurosci 2:246–253

Vaccarino FM, Schwartz ML, Raballo R, Rhee J, Lyn-Cook R (1999b) Fibroblast growth factor signaling regulates growth and morphogenesis at multiple steps during brain development. Curr Top Dev Biol 46:179–200

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415:1030–1034

Vincent A, Van Seuningen I (2009) Epigenetics, stem cells and epithelial cell fate. Differentiation 78(2–3):99–107
Visvanathan J, Lee S, Lee B, Lee JW, Lee SK (2007) The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. Genes Dev 21:744–749
Viti J, Gulacsi A, Lillien L (2003) Wnt regulation of progenitor maturation in the cortex depends on Shh or fibroblast growth factor 2. J Neurosci 23:5919–5927
Voigt T (1989) Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. J Comp Neurol 289:74–88
Wang Y, Yang J, Zheng H, Tomasek GJ, Zhang P, McKeever PE, Lee EY, Zhu Y (2009) Expression of mutant p53 proteins implicates a lineage relationship between neural stem cells and malignant astrocytic glioma in a murine model. Cancer Cell 15:514–526
Warren N, Caric D, Pratt T, Clausen JA, Asavaritikrai P, Mason JO, Hill RE, Price DJ (1999) The transcription factor, Pax6, is required for cell proliferation and differentiation in the developing cerebral cortex. Cereb Cortex 9:627–635
Watanabe D, Suetake I, Tada T, Tajima S (2002) Stage- and cell-specific expression of Dnmt3a and Dnmt3b during embryogenesis. Mech Dev 118:187–190
Watanabe D, Uchiyama K, Hanaoka K (2006) Transition of mouse de novo methyltransferases expression from Dnmt3b to Dnmt3a during neural progenitor cell development. Neuroscience 142:727–737
Wenning GK, Odin P, Morrish P, Rehncrona S, Widner H, Brundin P, Rothwell JC, Brown R, Gustavii B, Hagell P, Jahanshahi M, Sawle G, Bjorklund A, Brooks DJ, Marsden CD, Quinn NP, Lindvall O (1997) Short- and long-term survival and function of unilateral intrastriatal dopaminergic grafts in Parkinson's disease. Ann Neurol 42:95–107
Wine-Lee L, Ahn KJ, Richardson RD, Mishina Y, Lyons KM, Crenshaw EB (2004) Signaling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord. Development 131:5393–5403
Wolpert L (1969) Positional information and the spatial pattern of cellular differentiation. J Theor Biol 25:1–47
Yoon K, Nery S, Rutlin ML, Radtke F, Fishell G, Gaiano N (2004) Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. J Neurosci 24:9497–9506
Zechner D, Fujita Y, Hülsken J, Müller T, Walther I, Taketo MM, Crenshaw EB, Birchmeier W, Birchmeier C (2003) beta-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. Dev Biol 258:406–418
Zhang ZG, Chopp M (2009) Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic. Lancet Neurol 8:491–500
Zhang RL, Zhang ZG, Lu M, Wang Y, Yang JJ, Chopp M (2006) Reduction of the cell cycle length by decreasing G1 phase and cell cycle reentry expand neuronal progenitor cells in the subventricular zone of adult rat after stroke. J Cereb Blood Flow Metab 26:857–863
Zhang CL, Zou Y, He W, Gage FH, Evans RM (2008a) A role for adult TLX-positive neural stem cells in learning and behaviour. Nature 451:1004–1007
Zhang RL, Zhang ZG, Chopp M (2008b) Ischemic stroke and neurogenesis in the subventricular zone. Neuropharmacology 55:345–352
Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132:645–660
Zhu J, Zhou L, XingWu F (2006) Tracking neural stem cells in patients with brain trauma. N Engl J Med 355:2376–2378
Zuccato C, Cattaneo E (2009) Brain-derived neurotrophic factor in neurodegenerative diseases. Nat Rev Neurol 5:311–322

Chapter 13
Liver Stem Cells

Tohru Itoh, Hinako Takase, Minoru Tanaka, and Atsushi Miyajima

Abstract The liver is an essential organ for life, serving as the center for metabolism and playing various critical functions in controling systemic homeostasis. Among multiple types of cells comprising the liver, hepatocytes and cholangiocytes are the two epithelial cell lineages in the organ and commonly originate from hepatoblasts during organogenesis in the developing embryos. Thus, hepatoblasts possess bi-lineage differentiation potential into hepatocytes and cholangiocytes, a phenotypic feature that can best distinguish and define liver stem cells. Although the liver is considered not to rely on any resident stem cell population for their homeostatic maintenance, facultative stem/progenitor cells with the bi-lineage differentiation potential, referred to as oval cells in rodents, do emerge under severe damage conditions and contribute to the regenerative processes. Identification of specific markers has enabled researchers to isolate and characterize these fetal and adult stem/progenitor cell populations. *In vitro* culture systems as well as *in vivo* studies using animal models have been elucidating detailed molecular mechanisms, including intercellular signaling webs and intracellular transcriptional regulatory networks, that coordinately regulate development, differentiation and behavior of these cells. Understanding the cellular and molecular basis of liver development and regeneration from the perspective of the embryonic and adult stem/progenitor cells should make invaluable contributions to future development of technologies to produce fully functional hepatocytes *in vitro* that are applicable for cell therapy and pharmaceutical screening.

T. Itoh, Ph.D. (✉) • M. Tanaka, Ph.D. • A. Miyajima, Ph.D.
Laboratory of Cell Growth and Differentiation, Institute of Molecular and Cellular Biosciences, The University of Tokyo,
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
e-mail: itohru@iam.u-tokyo.ac.jp

H. Takase, Ph.D.
Department of Developmental Biology, Howard Hughes Medical Institute, School of Medicine, Stanford University, Stanford, CA 94305-5323, USA

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_13,

13.1 Introduction

In the adult human, the liver is the largest organ in the body, accounting for around one fiftieth of the body weight. The mammalian liver plays multiple critical roles in maintaining vital activity of the organisms, including metabolism of amino acids, lipids, and carbohydrates, serum protein synthesis, detoxification of xenobiotic compounds, production and secretion of bile, immune regulation, and so forth. To achieve these complex biological functions, the liver possesses a characteristic and sophisticated structure composed of several different types of cells (Fig. 13.1). Hepatocytes, also known as the liver parenchymal cells, account for 80% of the total volume of the organ and serve as the principal cell type to execute the majority of the organ's functions. The other cell types, collectively termed as non-parenchymal cells (NPCs), include cholangiocytes (bile duct epithelial cells), Kupffer cells, hepatic stellate cells, endothelial cells, coelomic epithelial cells (mesothelial cells), and several kinds of immune cells. While each of these cell types has its own embryonic origin (Asahina et al. 2011), hepatocytes and cholangiocytes, the two epithelial lineages in the organ, derive from a common precursor cell population, so-called hepatoblasts, in the developing liver (Lemaigre 2009; Tanaka et al. 2011; Tanimizu and Miyajima 2007; Zhao and Duncan 2005) (Fig. 13.2a). Thus, the term "liver stem cell" (or "hepatic stem cell") is most generally applied to represent this type of bi-potential progenitor cells that can differentiate to both hepatocytes and cholangiocytes. Hepatoblasts, however, are usually considered a cell population found only during the fetal period, and it is not clear whether and how these cells are related to the putative stem/progenitor cell populations in the adult liver. In other words, the self-renewal capacity of hepatoblasts *in vivo* remains undetermined. Thus, it would be safer to denote these cells as the fetal liver "stem/progenitor" cells, and we would like to adopt this description in this chapter.

In contrast to the situation in the developing liver, where hepatoblasts are fairly well established as the bipotential liver stem/progenitor cell, the one regarding the adult liver still has considerable controversy. In many other organs, such as the hematopoietic and epidermal systems and the small intestine, the tissue stem cells can be defined, and have indeed been isolated and/or anatomically located, as the cells that are responsible for normal tissue turnover. Thus, those stem cells, under the physiological condition, continue self-renewal while producing the progenies that give rise to the mature cell types and eventually replace the expired cells in the organ to maintain homeostasis. In the liver, hepatocyte turnover occurs very slowly, and it is still unclear and under debate whether such a kind of "stem cell" also exists and is actively involved in homeostatic maintenance of the organ.

Nevertheless, apart from this relatively complicated situation regarding the *bona fide* stem cell in the adult liver, researchers in this field have been quite successful in identifying and characterizing several different classes of putative adult liver stem cells, which should be of significant importance particularly in view of therapeutic applications. Classification of these different adult liver stem cells will be briefly described in Sect. 13.2.2.

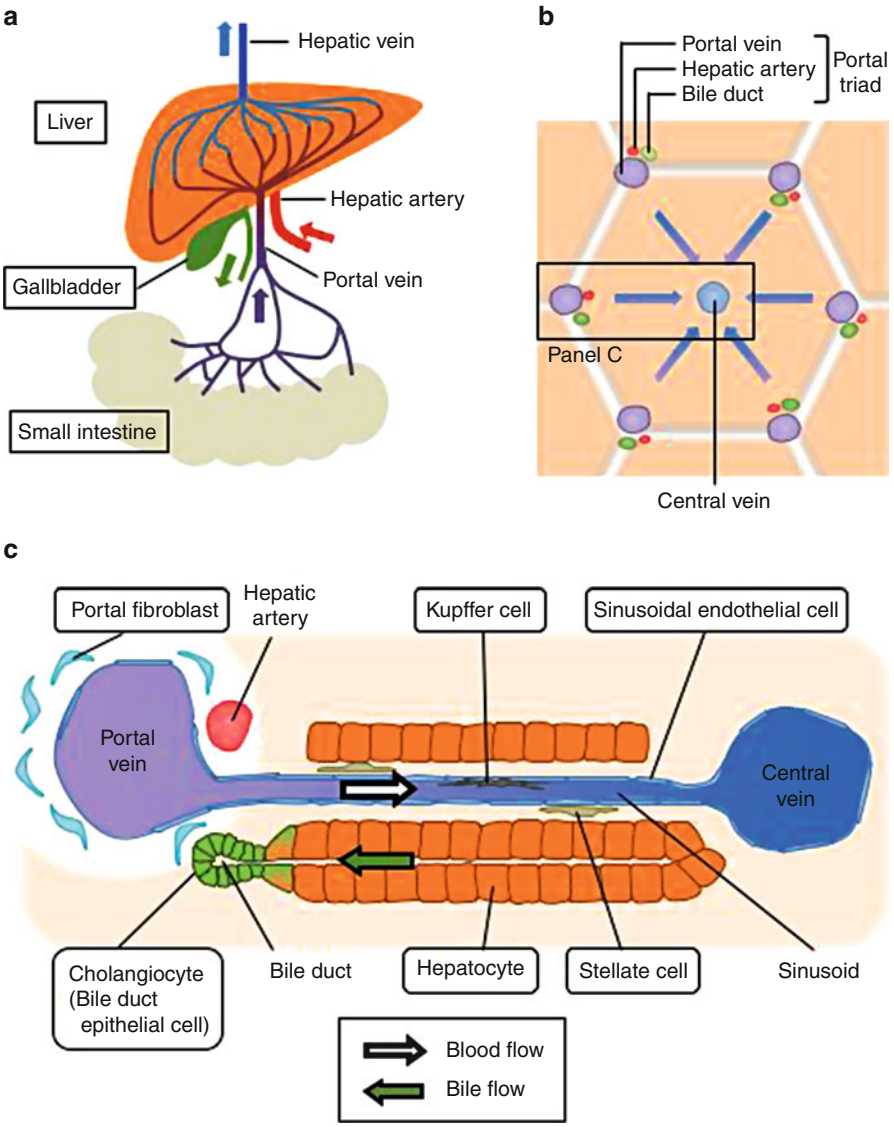


Fig. 13.1 Architecture of the liver. The liver has a dual blood supply via the portal vein and the hepatic artery. The portal vein delivers the venous blood flowing from the intestines, pancreas and spleen, while the hepatic artery supplies oxygen to the liver (**a**). The liver is composed of multiple functional units, called liver lobules, and at each of their corners there is a portal triad of vessels consisting or the portal vein, the hepatic artery, and the bile duct (**b**). The liver lobule consists of plates of hepatocytes lined by sinusoidal capillaries that radiate towards the central vein (**c**). The bile produced in hepatocytes is collected in bile canaliculi, which connect to bile ducts. Bile ducts leads to the gallbladder and eventually to the duodenum, where the bile is excreted. The liver is composed of several different types of cells, among which hepatocytes and cholangiocytes are the epithelial lineages

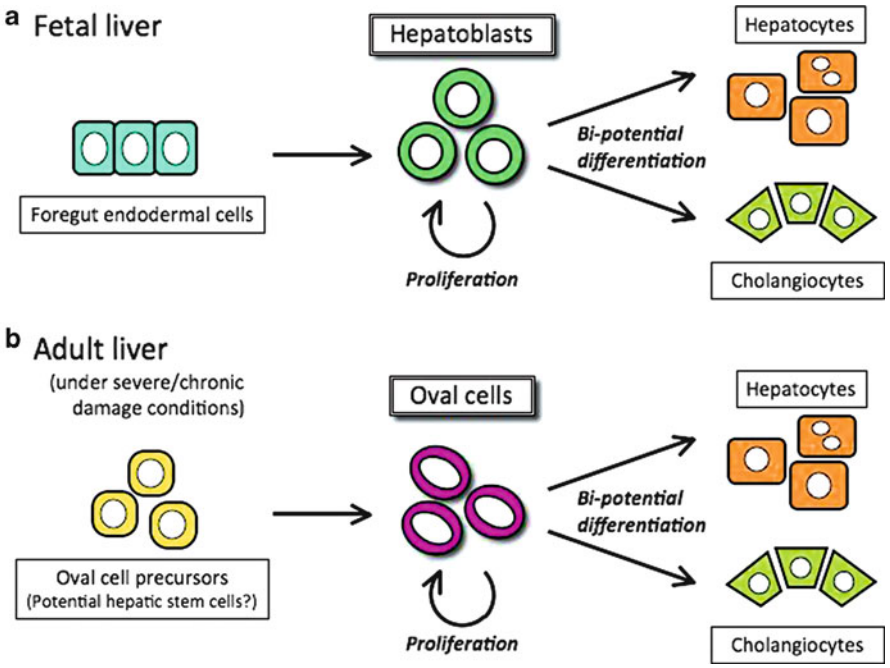


Fig. 13.2 Stem/progenitor cell populations in the fetal and adult livers. In the course of fetal liver development (**a**), hepatoblasts are derived from foregut endodermal cells, proliferate, and then undergo differentiation into two epithelial cell lineages, hepatocytes and cholangiocytes (bile duct epithelial cells). This bi-lineage differentiation potential is regarded as the hallmark of liver stem/progenitor cells. In the adult liver, regeneration can usually be achieved by replication of differentiated, mature cell populations (not shown here). Under severe/chronic liver damage conditions (**b**), however, facultative stem/progenitor cells, called oval cells in rodents, emerge from hitherto unidentified precursor cells and expand. These cells also possess bi-lineage differentiation potential and are considered to contribute to the regeneration process

13.2 Derivation/Classification

13.2.1 Hepatoblasts

Among the three germ layers generated during gastrulation (i.e., ectoderm, mesoderm, and endoderm), the liver derives principally from the endoderm. The endoderm differentiates into the primitive gut, which in turn gives rise to the gastrointestinal tract as well as various associated organs, including the thyroid, lung, pancreas, as well as liver. During the early stage of the liver development, interaction between the endoderm and the adjacent mesoderm plays a key role in induction of the organ (Fig. 13.3).

The processes and mechanisms of mammalian liver development have been most extensively studied in the mouse embryos. Liver organogenesis starts at embryonic day

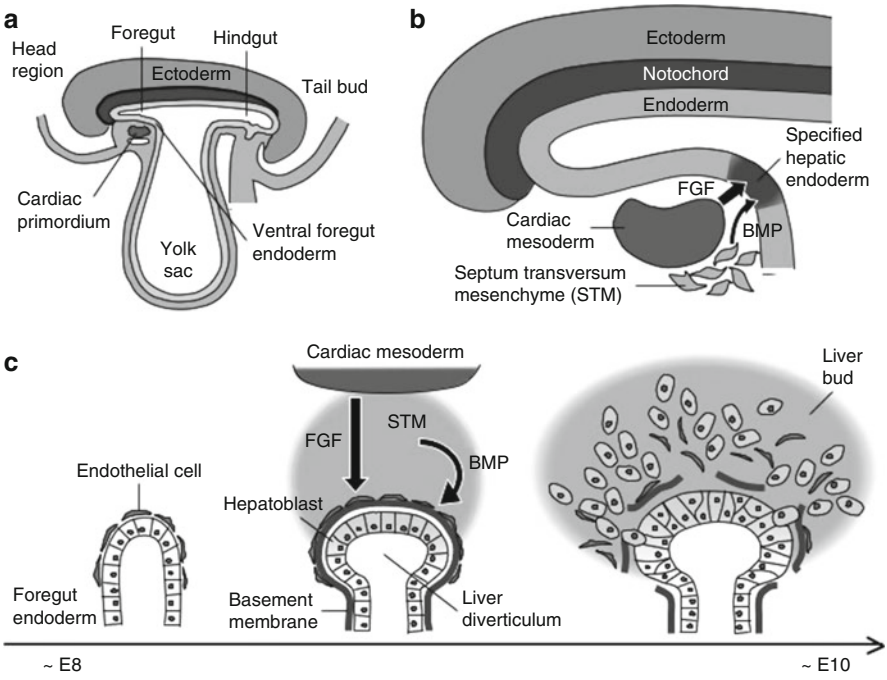


Fig. 13.3 Origin and early development of the liver. In the developing mouse embryo, the liver starts to form from the ventral foregut endoderm (**a**). Inductive paracrine signals from the adjacent cardiac mesoderm and the septum transversum mesenchyme (STM) together drive hepatic fate specification in a part of the ventral foregut endoderm (**b**), leading to generation of hepatoblasts, the fetal liver stem/progenitor cells. Hepatoblasts undergo dynamic morphological changes, and then delaminate and migrate into the STM to form the liver bud (**c**)

(E) 8.0–8.5 in mice, which corresponds to about 3 weeks of gestation in humans. At this stage, the ventral foregut endoderm, a part of the endoderm from which the liver forms, faces the developing heart and receives inductive signals for the hepatic fate from the cardiac mesoderm. The fibroblast growth factor (FGF) family of secreted proteins has been shown to mediate the inductive signals (Jung et al. 1999). In addition, septum transversum mesenchyme (STM), a mesodermal tissue locating adjacently to both of these organ primordia, also contributes to hepatic fate induction by providing another soluble factor, bone morphogenetic protein (BMP) (Rossi et al. 2001). Coordinated action of both FGF and BMP drives the liver developmental program with concomitant induction of several hepatic lineage-specific genes, such as *Albumin* and *Transthyretin*. This process is called "hepatic specification" and leads to generation of hepatoblasts, the fetal liver stem cells, initially lining up to form the hepatic endoderm.

Soon after the hepatic endoderm formation, hepatoblasts undergo dynamic changes in their morphology and localization, the process that can be divided into three stages (Bort et al. 2006). In stage I (E8.5), along with the expression of

liver-specific genes, the initially cuboidal hepatoblasts become columnar in shape, leading to the formation of a thickened epithelium. In stage II (E9.0–E9.5), the hepatoblasts further change their morphology to become a pseudo-stratified epithelium. A study using mice deficient of the hematopoietically expressed homeobox (Hhex, or Hex) gene has shown that this transcription factor is critical for this stage (Bort et al. 2006). In stage III (E9.5+), the basal lamina that has covered the epithelium breaks down and the hepatoblasts start to delaminate and then migrate into the surrounding stroma, the STM, to form the liver bud. This step is also controlled by the functions of homeobox transcription factors, prospero-related homeobox 1 (Prox1), hepatocyte nuclear factor (HNF) 6 (HNF6; also known as Onecut-1 or Oc1) and Onecut2 (Oc2) (Margagliotti et al. 2007; Sosa-Pineda et al. 2000).

After the liver bud formation, hepatoblasts continuously proliferate throughout the embryonic days. Proliferation and survival of hepatoblasts is known to be regulated by various extracellular signals, such as hepatocyte growth factor (HGF), transforming growth factor beta (TGFbeta), Wnt/beta-catenin, and Sonic hedgehog (Hirose et al. 2009; Micsenyi et al. 2004; Schmidt et al. 1995; Tanimizu and Miyajima 2007; Weinstein et al. 2001). These signals may act on hepatoblasts either in an autocrine fashion, or by being supplied from the surrounding mesenchymal cells. Endothelial cells also play a critical role in hepatoblast regulation, as mice lacking endothelial cells show a defect in liver bud outgrowth (Matsumoto et al. 2001). The molecular nature of the signal provided by endothelial cells still remains elusive.

13.2.2 Adult Liver Stem/Progenitor Cells

In the field of liver biology, the term “liver stem cells” (or “hepatic stem cells”) have been defined and used by researchers in several different ways. As exemplified by Grompe (2003), the definitions can include, but may not be limited to, the following:

(a) Cells responsible for normal tissue turnover
(b) Cells which give rise to regeneration after partial hepatectomy (PH)
(c) Cells responsible for progenitor-dependent regeneration
(d) Transplantable liver repopulating cells
(e) Cells which result in hepatocyte and bile duct phenotype *in vitro*

As is the case with stem cells in several other tissues/organs, clonogenic potential (colony forming activity) *in vitro* and long-term label-retaining activity *in vivo* have also been utilized as hallmarks to identify putative liver stem cells. Notably, these definitions are not mutually exclusive, and a given “liver stem cell” population may fulfill some of them simultaneously. For each definition, stem cells are accredited according to different and specific types of assays, either *in vitro* or *in vivo*.

Hepatocyte turnover under the physiological condition is relatively slow, which makes it quite difficult to investigate the cellular behavior in the course of homeostatic maintenance of the liver. Nevertheless, hepatocyte replacement does occur,

and there must be some mechanism that ensures this tissue turnover. One of the long-standing model is the so-called "streaming liver hypothesis", where a "flow" of hepatocytes is assumed, just analogous to the well-appreciated crypt-to-villi movement of the intestinal epithelial cells. In this model, hepatocytes are newly formed in the periportal region and then gradually move, while undergoing lineage maturation, toward the central vein (Zajicek et al. 1985). Although appealing, much of the evidence accumulated so far argues against this hypothesis, and it is more favorably considered that the liver maintenance is rather achieved by simple division of the preexisting hepatocytes. Interestingly, a study in the human liver using mitochondrial mutations as a genetic marker identified that clonal patches of hepatocytes did emerge from the periportal regions and extended toward the central veins, supporting the presence of the hepatocyte flow as assumed by the streaming liver hypothesis (Fellous et al. 2009). Moreover, a recent genetic lineage-tracing study in mice has provided striking evidence supporting the hypothesis. In those experiments, SRY-box containing gene 9 (Sox9)-CreERT2 knock-in mice was crossed with a reporter strain where Cre-mediated recombination enables permanent cell labeling and subsequent fate tracking, and cholangiocytes were specifically pulse-labeled in adulthood by tamoxifen-dependent transient Cre activation. While lineage-labeled cells were initially confined to bile ducts, they gradually spread out to hepatocytes from the periportal toward pericentral regions as time proceeded and, after around 1 year, occupied the whole parenchyma nearly completely. The labeled cells also remained present in bile ducts, thereby indicating that the Sox9-expressing biliary cells can continuously supply mature hepatocytes for normal tissue turnover while possessing self-renewing activity as well (Furuyama et al. 2011). However, genetic lineage-tracing studies in mice by other groups, using a different Sox9-CreERT2 strain to label biliary cells or a Cre-expressing adeno-associated viral vector to specifically label hepatocytes, have both provided rather conflicting results with the above study and thus strongly argue against the streaming liver hypothesis (Carpentier et al. 2011; Malato et al. 2011). Further studies are needed to solve this seeming discrepancy and elucidate the exact nature and the underlying mechanisms for physiological maintenance of the liver.

The characteristic feature of the liver is its unique and remarkably high capacity to regenerate upon various injuries, such as those caused by partial hepatectomy or toxic insults. In rodent models, for example, after 70% partial hepatectomy, the liver can completely recover its initial volume and function within a week or so. During this recovery process, hepatocytes, as well as cholangiocytes, in the remaining liver undergo a few cycles of cell division to sufficiently restore the lost tissue (Michalopoulos and DeFrances 1997). Thus, the liver regeneration can usually be achieved by proliferation of the differentiated, postmitotic hepatocytes that remain intact, without necessitating an involvement of stem/progenitor cell populations. When the liver suffers from severe and/or chronic damages, however, hepatocyte proliferation is suppressed. It is under this condition when the facultative stem/progenitor cells are known to emerge and contribute to the liver regeneration process. Those stem/progenitor cells, referred to as oval cells in rodent models, are characterized by their potentials to proliferate as well as to differentiate into both

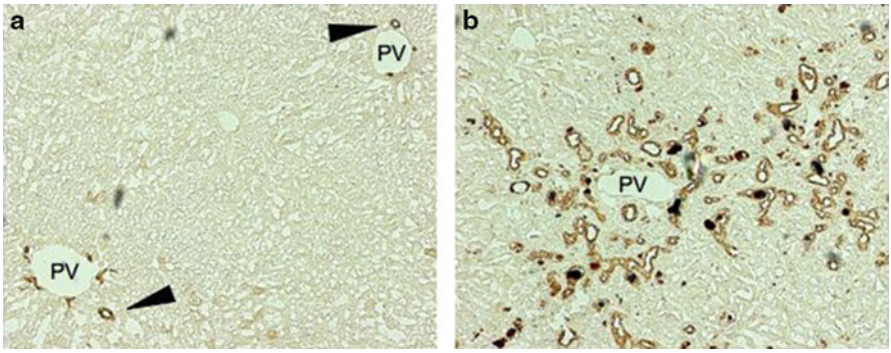


Fig. 13.4 Emergence of oval cells in a mouse model of chronic liver injury. Sections of the liver prepared from a normal mouse (**a**) and a mouse fed DDC diet for 8 weeks (**b**) were immunostained with anti-CK19 antibody. In the liver of the mouse fed DDC diet, CK19+ oval cells emerge from the periportal area, forming duct-llike structures (b; brown signals). Note that CK19 marks cholangiocytes comprising bile ducts in the normal liver (a; arrowheads). PV, portal vein

hepatocytes and cholangiocytes, the two epithelial lineages in the liver (Duncan et al. 2009; Grompe 2003; Matthews and Yeoh 2005; Newsome et al. 2004; Tanaka et al. 2011; Tanimizu and Miyajima 2007) (Fig. 13.2b). The most popular model to induce oval cells is the 2-acetylaminofluorene (2-AAF)/PH system in rats, where hepatocyte proliferation is blocked by 2-AAF prior to PH. This model, however, is not applicable to induce oval cells in mice. Other procedures, such as the administration of a 3,5-diethoxycarbonyl-1,4-dihidro-collidine (DDC)-containing diet (Preisegger et al. 1999) and a choline-deficient ethionine-supplemented diet (CDE) (Akhurst et al. 2001) have been established and used in mice, as well as in rats (Fig. 13.4). Notably, most of the experimental procedures used to induce oval cell emergence and proliferation in the rodent liver eventually lead to tumorigenesis.

Although the term "oval cells" are used specifically in rodents, cells with similar characteristics have also been reported in various human liver diseases, such as chronic viral hepatitis, alcoholic liver disease (ALD), and nonalcoholic fatty liver disease (NAFLD), and are also implicated in tumorigenesis (Fausto 2004; Lee et al. 2006; Roskams et al. 2003). In humans, these cells are usually referred to as "hepatic progenitor cells" or "intermediate hepatobiliary cells".

While oval cells are well known to emerge always from the periportal area, the cellular origin of oval cells is still not clarified (Fig. 13.5). Ever since their initial characterization, phenotypic resemblance between oval cells and bile duct epithelial cells has suggested that they presumably originate from the biliary tree. The fact that most of the molecular markers for oval cells are also expressed in cholangiocytes supports this notion. It is not clear, however, whether most if not all cholangiocytes can equally or similarly behave as progenitors for oval cells, or there is a certain type of specialized "cell-of-origin for oval cells" located somewhere among cholangiocytes. Potentially lying on an extension of the latter possibility is the model that the canal of Hering, a structure where interlobular bile ducts and

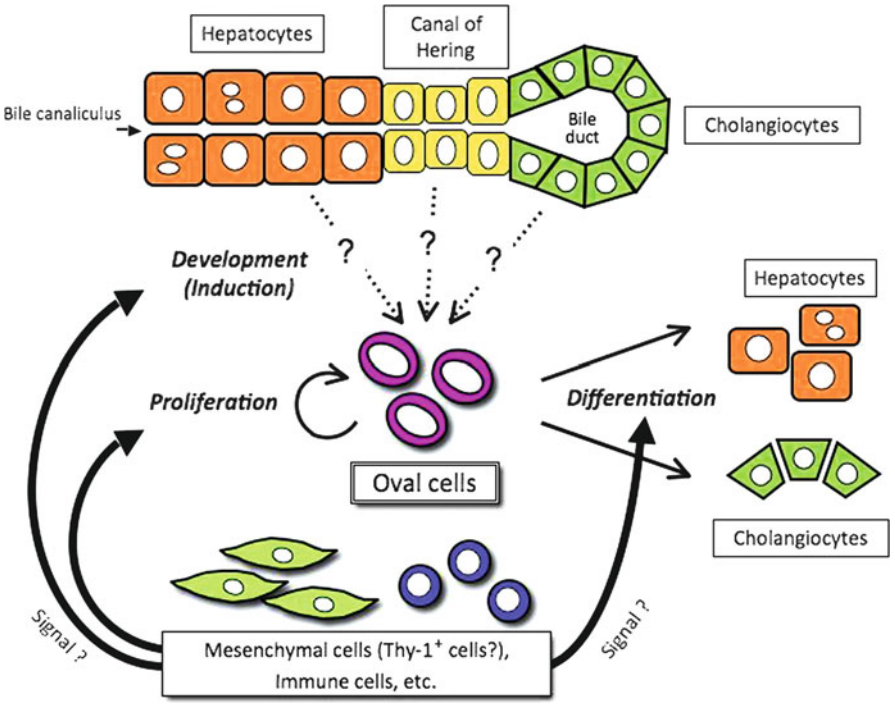


Fig. 13.5 Relationship among cells involved in oval cell response. Upon severe/chronic liver damages, oval cells emerge from the periportal region. The exact origin of oval cells has not yet been identified, but is supposed to be the canals of Hering, cholangiocytes, or heptocytes. Together with oval cells, several types of mesenchymal cells as well as immune cells accumulate in the injured liver and are often observed surrounding oval cells. These cells are likely to modulate induction, proliferation, migration, and/or differentiation of oval cells by means of various signaling mechanisms including direct cell-cell interaction, secretion of soluble factors (cytokines), and deposition of extracellular matrices, thereby playing key roles in regulation of oval cell response

hepatocytes are connected, is the origin of oval cells (Paku et al. 2001). Given its anatomical location in between cholangiocytes and hepatocytes, it appears reasonable to assume that this structure may serve as a niche for putative stem cells for these two cell lineages. Unfortunately, a direct proof for this model is hampered by lack of any specific marker for cells constituting the canal of Hering. Identification of such a molecule and a subsequent genetic lineage-tracing study should help clarifying this issue. Although a possible contribution of hepatocytes as an origin of oval cells can also be considered, a study using mice with chimeric livers have suggested that this is not likely the case (Wang et al. 2003).

As a matter of course, emergence and expansion of oval cells upon liver injury is not an autonomous process within these cells but involves various other types of cells, which interact either directly or indirectly with oval cells, and also possibly with their putative precursor cells. Thus, they together shape the entire phenomenon

often termed as “oval cell response”. Mesenchymal cells such as stellate cells have long been suggested to physically interact with oval cells and exert some signals on them. A recent study has highlighted that a population of mesenchymal cells expressing thymus cell antigen 1 (Thy-1), which is distinct from stellate cells or myofibroblasts, reside in close proximity to oval cells in rat liver (Yovchev et al. 2009). Further characterization of this unique population may provide a clue to understand the nature of signals controlling oval cell behaviors. Chronic injury conditions in the liver are usually associated with induction of inflammation, and the role of lymphocytes and inflammatory responses have also been suggested (Knight et al. 2007; Strick-Marchand et al. 2008). In accord with this notion, several inflammatory cytokines, such as tumor necrosis factor (TNF)-alpha and interferon-gamma, have been shown to modulate oval cell response, although their modes of action remain not fully clarified. A cytokine well appreciated to be involved in oval cell regulation is TNF-like weak inducer of apoptosis (TWEAK); transgenic mice overexpressing TWEAK in the liver exhibit periportal oval cell hyperplasia, while DDC diet-induced oval cell expansion was significantly reduced in mice lacking the Tweak receptor Fn14 as well as in wild-type mice administrated with a blocking anti-TWEAK monoclonal antibody (Jakubowski et al. 2005). As a signal related to oval cell response, several studies have identified activation of the canonical Wnt/beta-catenin pathway in oval cells (Apte et al. 2008; Hu et al. 2007; Itoh et al. 2009; Yang et al. 2008). The Wnt/beta-catenin pathway is well known to be involved in stem cell regulation in various organs and tissues, and further characterization of the role of this pathway in oval cells, including its relevant target genes and interaction with other signaling pathways, is awaited.

13.3 Characteristics/Properties

13.3.1 Hepatoblasts

In order to characterize a particular cell population, the cell sorting method using antibodies against specific surface markers expressed on that population is a powerful tool, as the cells can be viably isolated and thus can be subjected to *in vitro* culture and/or *in vivo* transplantation experiments. In the last decade, much effort has been made to identify such specific cell surface antigens expressed on fetal hepatoblasts, leading to successful identification of several markers as well as establishment of protocols to isolate and culture these cells.

Delta-like 1 homolog (Dlk1; also known as Pref-1 or fetal antigen 1) was initially identified as a marker for mouse hepatoblasts, and has later been shown to be useful to enrich and purify rat fetal liver progenitor cells with the liver repopulating activity (Oertel et al. 2008; Tanimizu et al. 2004). DLK1 is also known to

be expressed in human fetal liver (Floridon et al. 2000). In mouse embryos, Dlk1 expression in the liver is initially observed around E9.0 in the developing liver bud and is maintained at a high level until E16, which then declines significantly and disappears at the neonatal and adult stages. Dlk1$^+$ cells isolated from E14.5 livers expressed albumin and formed colonies composed of hetpatocyte (Albumin$^+$) and cholangiocyte (Cytokeratin [CK] 19$^+$) lineages *in vitro*. Moreover, 7% of the colony-forming Dlk1$^+$ cells formed large colonies containing more than 100 cells during 5 days of culture, thus indicating that Dlk1 serves as a useful marker to enrich highly proliferative, bipotential hepatoblasts from fetal liver.

E-cadherin is also widely used as a fetal hepatoblast marker (Nitou et al. 2002), although its expression is not necessarily limited in these cells but persists even in the differentiated epithelial lineages. Using a specific monoclonal antibody against E-cadherin, hepatoblasts could be isolated form E12.5 mouse liver with 90% purity and 40% yield. Other hepatoblast markers include Liv-2 in the mouse, whose antigen has not yet been molecularly identified (Watanabe et al. 2002).

In addition to these relatively simple isolation of hepatoblasts based on the expression of single positive selection markers, several other groups have established well elaborated protocols employing combinations of multiple markers, sometimes including those for negative selection. In the rat system, the RT1A1$^-$ OX18^{low} ICAM1$^+$ fraction of E13 fetal liver has been shown to contain hepatoblasts (Kubota and Reid 2000). Suzuki et al. designated as "hepatic colony-forming unit in culture (H-CFU-C)" a putative self-renewing stem cell population in the developing liver. Thus, based on an *in vitro* single cell-based assay of sorted cells, clonogenic cells capable of both self-renewal and multilineage differentiation were sought to be identified. They separated fetal liver cells based on expression of several markers including alpha6- and beta1-integrin subunits (CD49f and CD29, respectively) and demonstrated that the CD45$^-$ Ter119$^-$ c-Kit$^-$ CD29$^+$ CD49f$^+$ and CD45$^-$ Ter119$^-$ c-Kit$^-$ c-Met$^+$ CD49f$^{+/low}$ fractions of E13.5 mouse liver contained the H-CFU-C activity (Suzuki et al. 2000, 2002). Recently, CD13 (aminopeptidase N) has been identified as a surface marker expressed on a subset of the Dlk1+ hepatoblasts. Colony formation assays has revealed that clonogenic liver stem/progenitor cell activity can be enriched in the CD13+ fraction, compared with the Dlk1+ one (Kakinuma et al. 2009).

Using a combination of surface markers, a recent study has elucidated the phenotypic transition of hepatoblasts in the course of mouse fetal liver development (Tanaka et al. 2009). Thus, upon liver bud formation at around E9, hepatoblasts expressing both Dlk1 and epithelial cell adhesion molecule (EpCAM; also known as CD326, Tacstd1, or Trop1), a known marker for cholangiocytes and oval cells (see below), emerge from EpCAM$^+$Dlk1$^-$ foregut endodermal cells. The EpCAM$^+$Dlk1$^+$ cells contain highly proliferative hepatoblasts at E11.5, and thereafter undergo dramatic reduction in expression of EpCAM concomitantly with losing proliferative potential. At around E16.5, EpCAM expression is upregulated in ductal plates around the portal vein, while absent in immature hepatocytes.

13.3.2 Adult Liver Stem/Progenitor Cells

Oval cells were initially described by Farber, using a rat model of liver carcinogenesis, as "small oval cells about the ducts and vessels in the portal areas" having "scanty, lightly basophilic cytoplasm and pale blue-staining nuclei (by hematoxylin and eosin stain)" (Farber 1956). Since then, many studies have further characterized these cells and have established them as facultative liver stem/progenitor cells that are likely to play a relevant role in liver regeneration from various types of injuries (Duncan et al. 2009; Grompe 2003; Matthews and Yeoh 2005; Newsome et al. 2004; Tanaka et al. 2011; Tanimizu and Miyajima 2007). Thus, oval cells are considered to be capable of differentiating into two hepatic epithelial lineages, i.e., hepatocyte and cholangiocyte. In possible relation to this notion, oval cells express both hepatocyte (Albumin) and cholangocyte (CK19) markers. The immature hepatocyte marker alpha-fetoprotein (Afp) is known to be expressed in oval cells in rats, but not in mice (Jelnes et al. 2007). Similarly, expression of the hepatoblast marker Dlk1 has been shown in a subpopulation of rat oval cells, but is not found in mouse oval cells (Jelnes et al. 2007; Jensen et al. 2004; Tanimizu et al. 2004). There are several monoclonal antibodies that have long been used as "golden standards" to recognize oval cell markers, such as OV-1 and OV-6 in rats (Dunsford and Sell 1989) and A6 in mice (Engelhardt et al. 1990). OV-1 antibody reacts with an unknown antigen expressed on the surface of oval cells and thus can be used to isolate these cells, while OV-6 antibody recognizes a common epitope in the cytoskeleton components CK14 and CK19 (Bisgaard et al. 1993). Unfortunately, the A6 antibody used for mouse studies recognizes some intracellular antigen and thus is not suitable to be used for sorting of viable oval cells.

Similar to the situation with fetal liver hepatoblasts, much effort has been made in recent years to explore cell surface molecules that can be used to identify and isolate oval cells. This has led to the identification of EpCAM and CD133 (also known as prominin 1) as novel oval cell markers in both mice and rats (Okabe et al. 2009; Rountree et al. 2007; Suzuki et al. 2008b; Yovchev et al. 2007). The oncofetal protein glypican-3 has also been documented as a rat oval cell marker (Grozdanov et al. 2006). Notably, however, these molecules, as well as the OC-1/OC-6 and A6 antigens, are all expressed also in cholangiocytes in the normal liver. This fact strongly implies a close relationship between cholangiocytes and oval cells as mentioned earlier, with the former possibly being an origin of the latter.

Interestingly, Trop2 (Tacstd2), a transmembrane molecule that is structurally related to EpCAM, has been found to be expressed exclusively in oval cells in the injured liver, but not in cholangiocytes in the normal liver (Okabe et al. 2009). Thus, Trop2 may serve as a genuine "oval cell marker" and would be advantageous for further characterization of oval cells. Similarly, the transcription factor Foxl1 has been identified as another potential oval cell-specific marker (Sackett et al. 2009). Although this molecule is not a cell-surface antigen, a transgenic (Tg) mouse line expressing the Cre recombinase under the control of the Foxl1 promoter has been

made and proven to be quite useful. Thus, a lineage tracing study using this Foxl1-Cre Tg mice demonstrated that both hepatocytes and cholangiocytes were found as descendants of Foxl1$^+$ oval cells. This does not necessarily indicate that single oval cells can clonally differentiate into these two lineages, but strongly supports the notion that oval cells are bipotential progenitors for hepatocytes and cholangiocytes.

A recent study has established a panel of surface reactive monoclonal antibodies, including MIC1-1C3, that can each detect different populations of ductal and periductal cells in the mouse oval cell response (Dorrell et al. 2008). Intriguingly, some of them seem to label cell populations that are apparently enriched or reside specifically in the oval cell-induced livers, with little or no reactivity shown in the normal liver. Identification of the corresponding antigen molecules, as well as further characterization of these cell populations, should expedite our understanding of the mechanisms of the oval cell response at the cellular and molecular levels.

Using flow cytometry-based cell separation methods in combination with the aforementioned cell surface markers, oval cells can be viably isolated and subjected to *in vitro* culture to evaluate their proliferation and differentiation potentials. Studies based on the expression of EpCAM or CD133, and more recently of MIC1-1C3 or Foxl1-Cre-mediated fluorescent reporter, have consistently demonstrated that oval cells isolated from injured livers proliferate to form colonies *in vitro* in the presence of certain combinations of growth factors, and the clonally expanded cells are capable of differentiating into both hepatocyte and cholangiocyte lineages under appropriate culture conditions (Dorrell et al. 2011; Okabe et al. 2009; Shin et al. 2011; Suzuki et al. 2008b). These results strongly suggest that oval cells indeed possess clonal bi-lineage differentiation potential, at least *in vitro*, a notion which needs to be evaluated using *in vivo* experimental systems as well. As most of the oval cell antigens including EpCAM and CD133 are also expressed in cholangiocytes under uninjured conditions, the cells positive for these markers were also isolated from normal adult livers and similarly subjected to *in vitro* culture experiments (Kamiya et al. 2009; Okabe et al. 2009; Suzuki et al. 2008b). Interestingly, both EpCAM$^+$ cells and CD133$^+$ cells isolated from the normal liver also formed colonies as well as underwent differentiation into hepatocytes and cholangiocytes. Essentially the same results were also obtained with MIC1-1C3 (Dorrell et al. 2011). Thus, the normal adult liver harbors "potential hepatic stem cells", which can be defined as those with clonogenicity and bi-lineage differentiation potential *in vitro*, similar to H-CFU-C in the embryonic liver. Notably, EpCAM$^+$ cells isolated from human postnatal livers, as well as fetal livers, have also been found to contain closely related hepatic stem cells (hHpSCs) that can be defined *in vitro* (Schmelzer et al. 2007). The exact location and character of these potential hepatic stem cell populations *in vivo*, as well as their possible contribution to homeostasis and/or regenerative process of the liver, remain to be elucidated. In particular, it is tempting to speculate that these cells may serve as the precursors for oval cells, which needs to be addressed in future studies.

13.4 Differentiation Capacity and Their Precursors

As has been mentioned repeatedly in the preceding sections, the characteristic feature of the liver stem/progenitor cells is their potential to differentiate into two lineages, i.e., hepatocytes and cholangiocytes. In addition to these two hepatic cell lineages, much evidence has been accumulated supporting that the liver stem/progenitor cells are also capable of differentiating into pancreatic and other cell lineages both *in vitro* and *in vivo* under appropriate experimental settings. So far, circumstantial understanding of the mechanisms of liver stem/progenitor cell differentiation have been accomplished with regard to hepatoblasts in the developing liver, and herein we will focus mostly on this issue. Although oval cells have been shown to possess differentiation capacity to hepatocytes, cholangiocytes and other cell types, the underlying mechanisms have little been addressed.

13.4.1 Differentiation into Hepatocytes

By definition, hepatoblasts undergo during their development a fate decision between the hepatocyte and cholangiocyte lineages. The molecular basis of this hepato-biliary lineage decision still remains largely unknown. Several molecules have been implicated in differentiation of hepatoblasts into the cholangiocyte lineage, which will be discussed in the next section.

While the adult liver exerts various metabolic functions, the fetal liver lacks such functions and instead serves as a hematopoietic organ. Around E10 in the mouse embryo, hematopoietic stem cells immigrate into the fetal liver from the aorta-gonad-mesonephros region and the placenta, and expand their population tremendously in the microenvironment provided by the fetal liver till birth. During this period of time, hematopoietic cells enhance differentiation of hepatoblasts into hepatocytes by producing cytokines (Kinoshita et al. 1999). As hematopoiesis switches from the fetal liver to the bone marrow, liver organogenesis progresses to become a center for metabolism.

Several *in vitro* primary culture systems for fetal liver cells, and more specifically for sorted hepatoblasts, have been established and extensively used to characterize the cellular and molecular mechanisms of hepatocyte differentiation. In many cases, oncostatin M (OSM), one of the interleukin 6-famiy cytokines, shows potent activity to induce differentiation of hepatoblasts and immature hepatocytes to functional hepatocytes, as evidenced by expression of various hepatocyte-specific marker genes and acquisition of metabolic functions such as cytosolic glycogen accumulation and ammonia clearance from the culture medium (Kamiya et al. 1999). OSM transduces signals through a specific receptor complex containing the gp130 subunit, and the liver of mice lacking gp130 show defects in functional differentiation of hepatocytes (Kamiya et al. 1999). As OSM receptor-deficient mice exhibit no obvious anomaly in the liver development, other cytokines may play a similar or redundant

role (Tanaka et al. 2003). In addition to the OSM signals, HGF, extracellular matrices (ECMs), and cell-to-cell contacts have also been implicated in stimulating hepatocyte differentiation (Kamiya et al. 2002; Kojima et al. 2000; Suzuki et al. 2003). On the other hand, TNF-alpha has been shown to antagonize the differentiation-promoting activities of OSM and control the timing of hepatocyte maturation (Kamiya and Gonzalez 2004). Thus, TNF-alpha expression is detected in the liver until perinatal stages, and then decreases after birth. Concomitantly with this transition, hepatocytes are relieved from inhibitory effect of TNF-alpha and then strongly promoted to acquire mature metabolic functions.

In addition to these extracellular signals, hepatocyte differentiation and maturation are regulated by cell-intrinsic machineries involving various transcription factors. A set of transcription factors, such as HNF1alpha, HNF4, and CCAAT/enhancer binding protein (C/EBP) alpha, are known to be abundantly and characteristically expressed in hepatocytes and thus are collectively termed as "liver-enriched transcription factors". While studies using gene knockout mice have elucidated that each of these molecules has its own unique functions as manifested by observed specific phenotypes (Costa et al. 2003; Schrem et al. 2002, 2004), it has become evident that they function cooperatively to form a dynamic transcriptional network of autoregulatory and cross-regulatory loops (Kyrmizi et al. 2006; Lemaigre 2009). In addition, these liver-enriched transcription factors also interact with various other transcription factors and/or regulatory molecules in a context-dependent manner to achieve specific target gene expression. For example, C/EBPalpha is an essential factor for glucose metabolism during the perinatal stage, and mice lacking this transcription factor die soon after birth due to hypoglycemia caused by defective gluconeogenic gene expression (Wang et al. 1995). Despite of this specific functional requirement at the perinatal stage, C/EBPalpha is already expressed in E14.5 fetal liver, suggesting that an additional factor may function cooperatively to ensure its temporally-regulated activity. Indeed, the forkhead family transcription factor Foxo1 has been found to be inducibly expressed in the perinatal liver, physically interact with C/EBPalpha, and augment C/EBPalpha-dependent transcription of a gluconeogenic gene, phosphoenolpyruvate carboxykinase (PEPCK) (Sekine et al. 2007). On the other hand, C/EBPalpha is also critical for ammonia detoxification activity of hepatocytes, as the knockout mice lack expression of carbamoyl phosphate synthetase-I (CPS1), a key enzyme in the urea cycle, leading to hyperammonemia (Kimura et al. 1998). Again, CPS1 is expressed only after the neonatal stage, and hence an involvement of some regulatory factor for C/EBPalpha-dependent CPS1 expression was suspected. In this case, Y-box binding protein-1 (YB-1) has been identified to be a molecule that suppresses C/EBPalpha function and negatively regulates CPS1 expression in the fetal liver (Chen et al. 2009). YB-1 is highly expressed in E14.5 fetal liver, and the expression significantly declines before birth. This results in the release of C/EBPalpha from YB-1-mediated suppression on the CPS1 promoter, leading to expression of CPS1 and ammonia clearance activity. Taken together, transcriptional activities of C/EBPalpha are differently controlled by expression and cooperative function of specialized "gatekeeper" molecules, Foxo1 and YB-1, for gluconeogenic and urea cycle enzymes, respectively.

As hepatocytes acquire mature metabolic functions, they also undergo structural maturation and establish specialized tissue architectures that are associated with their functions (Tanimizu and Miyajima 2007). They construct so-called "hepatocyte-type" epithelial polarity, where the apical surface, termed the bile canaliculus, is formed between neighboring hepatocytes, and the polarized hepatocytes are organized to form a cord-like structure. A study using gene knockout mice has suggested that the small GTPase ARF6 plays a critical role in the latter process (Suzuki et al. 2006).

13.4.2 Differentiation into Cholangiocytes

Cholangiocytes are epithelial cells that line the biliary tract. The biliary tract can be separated ontogenetically into two parts, that is, the extrahepatic bile duct and the intrahepatic bile duct (IHBD). The extrahepatic bile duct is comprised of the hepatic ducts, the cystic duct, the common bile duct, and the gallbladder, and develops from the endoderm independently of the hepatoblast formation. On the other hand, cholangiocytes forming the intrahepatic bile ducts derive from hepatoblasts, as mentioned earlier. It is not clear how the extrahepatic and intrahepatic biliary tracts, developing separately, eventually anastomose.

The process of IHBD formation from hepatoblasts involves cholangiocyte differentiation (lineage specification) and morphogenesis of ductal structures. In mouse embryos, the initial sign of cholangiocyte specification can be recognized at E11.5, when the cholangiocyte marker Sox9 is expressed in liver cells that are located a short distance from the branches of the portal vein (Antoniou et al. 2009). These cells align around the portal vein to form a single-layered structure, called the ductal plate (Fig. 13.6). At E15.5, the ductal plate becomes focally bilayered to form the primitive ductal structures (PDS), and lumens can be detected between the two layers. A recent study has suggested that the PDS are transiently asymmetrical, in that the cells on the portal-side layer express Sox9 but not the hepatoblast marker HNF4, while that those on the parenchymal-side layer express HNF4 but not Sox9 (Antoniou et al. 2009). By E18.5, the hepatoblasts lining the parenchymal side of the PDS differentiate to cholangiocytes, leading to formation of radially symmetrical duct structures entirely delineated by cholangiocytes. During this process, the ductal plate cells that are not involved in tubulogenesis regress and eventually disappear, and the remaining ducts become surrounded by periportal mesenchymal cells. Although it has long been considered that this regression of ductal plate cells that do not contribute to the mature bile duct structure is mediated by apoptosis, a recent study has shown that they undergo not apoptosis but rather differentiation to a subset of hepatocytes in the periportal region (Carpentier et al. 2011).

With regard to the molecular mechanisms involved in cholangiocyte differentiation, the roles of several transcription factors have been implicated, such as Sal-like 4 (Sall4), T-box transcription factor 3 (Tbx3), the Onecut transcription factors HNF6 and OC2, and HNF1beta. Based on gene expression profile as well as overexpression

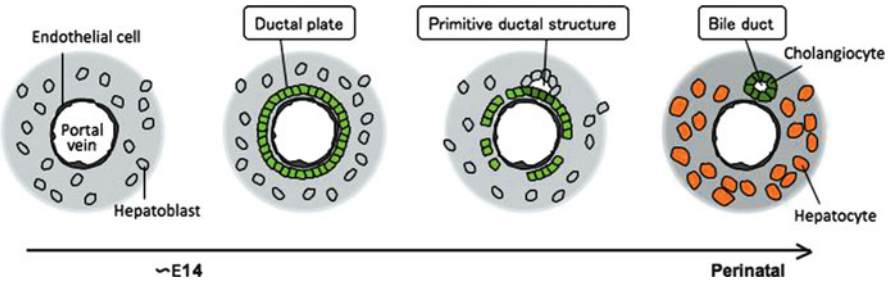


Fig. 13.6 Development of intrahepatic bile ducts in the fetal mouse liver. In the midgestation mouse liver, some hepatoblasts start to express biliary markers and these biliary precursor cells form a contiguous single-layered structure, called the ductal plate, around the portal vein. The ductal plate becomes focally bilayered to generate asymmetric primitive ductal structures (PDS), where the cells only on the portal-side layer express biliary markers. At around the perinatal stage, the hepatoblasts lining the parenchymal side of the PDS differentiate to cholangiocytes and symmetrical bile ducts are formed

and knockdown experiments in purified fetal mouse hepatoblasts, Sall4 has been shown to play a role in regulating the lineage commitment of hepatoblasts by inhibiting their differentiation into hepatocytes while driving the one toward cholangiocytes (Oikawa et al. 2009). In Tbx3 knockout mouse embryos, hepatoblast proliferation is severely impaired and biliary differentiation is promoted at the expense of hepatocyte differentiation, suggesting that Tbx3 plays a role in hepato-biliary lineage decision (Ludtke et al. 2009; Suzuki et al. 2008a). Gene expression analyses have shown that Tbx3 functions to maintain expression of hepatocyte transcription factors, HNF4alpha and C/EBPalpha, while suppressing that of cholangiocyte transcription factors, HNF6 and HNF1beta (Ludtke et al. 2009). Mice deficient of HNF6 shows bile duct malformation, and this phenotype is further enhanced by combined knockout of HNF6 and OC2 (Clotman et al. 2002, 2002). A direct and critical target of HNF6 is HNF1beta, and mice with liver-specific inactivation of HNF1beta shows defect in bile duct development (Coffinier et al. 2002). The Onecut transcription factors also regulate hepatobiliary lineage decision of hepatoblasts by modulating transforming growth factor (TGF) beta signaling. In the normal liver, TGFbeta signaling is found to be strongly activated near the portal veins but weakly in the rest of the parenchyma. In the liver of HNF6 and OC2 double knockout mice, increased TGFbeta signaling is observed in the parenchymal region, where "hybrid" hepatic cells that display characteristics of both hepatocytes and cholangiocytes are generated (Clotman et al. 2005). Thus, the Oncecut transcription factors play a role in shaping the proper gradient of TGFbeta signaling activity to ensure induction of cholangiocytes only in the periportal region.

Another molecular mechanism well known to be involved in bile duct formation is the Notch signaling pathway. In humans, mutations in JAGGED 1 (JAG1), a ligand for the Notch receptors, are associated with Alagille syndrome (ALGS or ALGS1; Online Mendelian Inheritance in Man #118450), an autosomal dominant disorder characterized by multiple developmental defects including neonatal cholestasis caused by a paucity of IHBD (Li et al. 1997; Oda et al. 1997). In addition, another

form of Alagille syndrome has been found to be caused by mutations in the NOTCH2 gene (ALGS2; Online Mendelian Inheritance in Man #610205) (McDaniell et al. 2006). In accord with these notions, mice doubly heterozygous for a Jag1 null allele and a Notch2 hypomorphic allele recapitulate most of the human syndrome phenotypes, including the bile duct paucity (McCright et al. 2002). A study using an *in vitro* culture of mouse hepatoblasts has shown that activation of the Notch signaling pathway promotes differentiation of hepatoblasts into the cholangiocyte lineage by coordinating a network of liver-enriched transcription factors including HNF1alpha and beta, HNF4, and C/EBPalpha (Tanimizu and Miyajima 2004). Indeed, conditional knockout of RBP-Jkappa, an essential downstream signal component of the Notch receptor, results in a reduced number of cholangiocytes at E16.5, confirming a role of this signaling pathway in cholangiocyte cell fate specification (Zong et al. 2009). In contrast, studies using the aforementioned compound (doubly heterozygous) mouse mutant for Jag1 and Notch2, or the liver-specific Notch2 knockout mice, have shown that the Notch2 signaling is rather required for bile duct morphogenesis, but is likely dispensable for biliary specification (Geisler et al. 2008; Lozier et al. 2008). Similarly, in fetal livers of mice lacking hairy and enhancer of split 1 (Hes1), a target of the Notch signaling, the ductal plate formation occurs normally but the subsequent remodeling and tubular structure formation is completely blocked (Kodama et al. 2004). The discrepancy may result from the presence of multiple Notch signaling components, including the ligands, receptors and targets, that can play redundant and compensatory roles in biliary differentiation. It is also possible that conditional deletion of Notch2 in the developing liver was not complete or early enough. In the periportal region of the developing liver, the ligand Jag1 is expressed in portal fibroblasts and the endothelium of the portal vein, as well as in cholangiocytes at later stages (Geisler et al. 2008; Kodama et al. 2004; Loomes et al. 2007; Lozier et al. 2008; Suzuki et al. 2008c; Zong et al. 2009). A recent study employing cell type-specific knockout mouse models has clearly demonstrated that deletion of Jag1 specifically in SM22alpha-expressing portal vein mesenchyme, but not in the endothelium, leads to the hepatic defects reminiscent of Alagille syndrome. In those mice lacking Jag1 in the SM22alpha-positive cells, the initial formation of the ductal plate occurs normally, yet those biliary-specified cells are unable to undergo subsequent morphological changes, leading to paucity of bile duct formation (Hofmann et al. 2010). Although the nature of the SM22alpha-expressing cells is not fully characterized, it is considered most plausible that portal fibroblasts stimulate cholangiocytes lining the ductal plate via the Jag1/Notch2 interaction and the downstream Hes1 expression, which leads to induction of ductal morphogenesis.

13.4.3 Differentiation into Non-hepatic Lineages

The liver and the pancreas share a common developmental origin, and a bipotential precursor cell population for these organs has been identified within the embryonic endoderm (Deutsch et al. 2001). In addition, hepatocytes and pancreatic beta-cells

are known to have similarities in gene expression profiles and possess similar inherent glucose sensing systems, thereby being capable of responding to changes in blood glucose concentrations. Consistent with these facts, many studies have demonstrated that liver stem/progenitor cells from both embryonic and adult origins as well as hepatocytes can be converted to insulin-producing cells, functional pancreatic beta-cell-like cells, and/or to islet-like cell clusters containing other pancreatic lineages under certain conditions.

Clonally expanded H-CFU-C derived from fetal mouse liver show expression of pancreatic endocrine and exocrine lineage markers in culture, and can be integrated into and form pancreatic ducts and acinar cells when transplanted into pancreas of recipient mice (Suzuki et al. 2002). Notably, H-CFU-C is also shown to be capable of differentiating into gastric and intestinal cells *in vivo*. Purified adult rat hepatic oval cells can be differentiated into pancreatic endocrine hormone-producing cells when cultured in a high-glucose environment (Yang et al. 2002). Rat liver epithelial WB cells, which represent the cultured counterpart of stem-like cells derived from normal adult liver, can be reprogrammed into functional insulin-producing cells by stable expression of pancreatic duodenal homeobox 1 (Pdx1) or its super-active form (Pdx1-VP16) (Tang et al. 2006). Epithelial progenitor cells derived from human fetal liver (FH cells) can also be induced to differentiate into insulin-producing cells after expression of the PDX1 gene (Zalzman et al. 2003).

In addition to these *in vitro* experiments, several studies employing *in vivo* gene delivery systems have shown that adenoviral vector-mediated transduction of pancreatic transcription factors, such as Pdx1, Neurogenin3 (Ngn3), NeuroD, and MafA, can induce formation of ectopic islet-like cells and production of insulin in the adult liver (Ferber et al. 2000; Kojima et al. 2003; Song et al. 2007; Wang et al. 2007). Although these phenomena have been considered to represent trans-differentiation of mature hepatocytes into pancreatic cells, a recent study employing Ngn3 gene transfer in combination with a genetic lineage tracing have suggested an alternative possibility. Thus, introduction of this transcription factor can sufficiently induce emergence of ectopic, periportal islet-like cell clusters in streptozotocin (STZ)-induced diabetic model mice, and these clusters do not originate from differentiated hepatocytes but are rather likely produced by "trans-determination" of oval cell-like progenitor cells, which are lineage-determined but not terminally differentiated (Yechoor et al. 2009). In view of this, it is noteworthy that DDC-induced activation of hepatic oval cells *in vivo* has been reported to ameliorate STZ-induced diabetes in mice (Kim et al. 2007).

13.5 Potential Application for Therapies

At present, orthotopic liver transplantation is the most commonly used procedure to treat various liver diseases. This, however, has always been hampered by persistent shortage of donor organs. Although isolated mature hepatocytes when transplanted have been shown to successfully repopulate the recipient liver with considerably high

efficiency at least in rodent models, the rates of engraftment and survival of transplanted hepatocytes in human liver is often very limited. Furthermore, despite that mature hepatocytes show tremendous proliferative activity in response to regenerative stimuli *in vivo*, they usually lose this capacity immediately once isolated and subjected for *in vitro* cultures. Thus, the ability to obtain an unlimited supply of human hepatocytes from any expandable source should significantly improve the development and clinical application of hepatocyte transplantation. In addition, it will also facilitate the studies on the basic mechanisms of human liver diseases, as well as evaluation of drugs for their actions and toxicities due to the metabolism of xenobiotics in hepatocytes. Considering the strong proliferative potential and amenability for *in vitro* manipulation, the liver stem/progenitor cells may be attractive candidates for these applications. These cells may also be useful for cell therapy to treat diabetic patients, given their potential to be effectively reprogrammed toward pancreatic lineages. However, isolation of fetal hepatoblasts and adult hepatic stem/progenitor cells from human liver for therapeutic use should be practically quite difficult.

In the last decade, much effort and concomitant progress have been made in establishing the protocols to generate various types of functionally differentiated cells, including mature hepatocytes, *in vitro* from pluripotent or multipotent stem cells (Snykers et al. 2009), particularly from embryonic stem (ES) cells (Agarwal et al. 2008; Basma et al. 2009; Cai et al. 2007) and more recently from induced pluripotent stem (iPS) cells (Gai et al. 2010; Si-Tayeb et al. 2010; Song et al. 2009; Sullivan et al. 2010). This has led to the notion that application of the precise conditions that recapitulate the normal developmental process within the embryo is generally the best way to achieve highly functional derivatives. Thus, to produce hepatocytes for example, these pluripotent stem cells can be sequentially induced to differentiate to the definitive endoderm, then the hepatic lineage cells with the character of hepatoblasts, and finally to functional hepatocytes, directed by the timed use of appropriate amounts and combinations of cytokines (Gouon-Evans et al. 2006). In view of this, studies elucidating the mechanisms of the normal liver organogenesis, and particularly of hepatoblast development and differentiation, should provide an important clue to future development of a better protocol to induce functional hepatocytes *in vitro*. In the opposite way, *in vitro* differentiation system from human ES or iPS cells to hepatocytes can offer a means of elucidating the mechanisms of human liver development *ex vivo* (DeLaForest et al. 2011). Notably, use of specific surface markers for hepatoblasts or other hepatic cells to enrich particular cell lineages in the course of induced differentiation should be advantageous to obtain hepatocytes with better quality and quantity. Moreover, this will also beneficial to eliminate undifferentiated stem cells that remain contaminated, as these cells may potentially cause tumors such as teratoma upon transplantation into recipients.

Upon the emergence of the iPS cell technology, the fact that terminally differentiated somatic cells can be sufficiently converted to a totally different, pluripotent state by a relatively small number of defined factors urged many researchers to test the possibility that they could also be reprogrammed directly to different cell lineages without going through a pluripotent intermediate. This approach, so-called

direct reprogramming, has indeed been shown to be quite promising with various target cell lineages, including hepatocytes. By forcedly introducing a few endodermal and hepatic transcription factors, two groups have independently demonstrated that mouse fibroblasts can be converted to hepatocyte-like cells (Huang et al. 2011; Sekiya and Suzuki 2011). Functionally, they are still not fully mature hepatocytes, yet are able to repopulate *in vivo* when transplanted into a mouse model. These "induced hepatocytes (iHep)" may provide an alternative, relatively simple platform to realize gene and cell therapy to treat liver diseases.

13.6 Conclusion and Future Development in Research

In recent years, considerable progress has been made in our understanding of the mechanisms of liver development at the cellular and molecular levels. Establishment of methods for *in vitro* culture of fetal liver cells, in particular the isolated hepatoblasts, as well as various mouse models with genetic modifications have invaluably contributed to identify and elucidate the role of genes involved in fetal liver development. Although characterization of adult liver stem/progenitor cells represented by oval cells has been less achieved up to the present compared to that of hepatoblasts, it has become much accelerated by the finding and availability of useful marker molecules. Further analysis on the extrinsic signals and the intrinsic genetic and epigenetic programs regulating these cell populations should lead to clarifying the molecular basis of liver regeneration, as well as its similarities and differences with that of liver development. In-depth understanding of the mechanisms governing these complicated and elaborated processes should definitely help establish a better protocol to generate functional hepatic cells amenable to therapeutic cell transplantation and pharmaceutical drug development.

References

Agarwal S, Holton KL, Lanza R (2008) Efficient differentiation of functional hepatocytes from human embryonic stem cells. Stem Cells 26:1117–1127

Akhurst B, Croager EJ, Farley-Roche CA, Ong JK, Dumble ML, Knight B, Yeoh GC (2001) A modified choline-deficient, ethionine-supplemented diet protocol effectively induces oval cells in mouse liver. Hepatology 34:519–522

Antoniou A, Raynaud P, Cordi S, Zong Y, Tronche F, Stanger BZ, Jacquemin P, Pierreux CE, Clotman F, Lemaigre FP (2009) Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 136:2325–2333

Apte U, Thompson MD, Cui S, Liu B, Cieply B, Monga SP (2008) Wnt/beta-catenin signaling mediates oval cell response in rodents. Hepatology 47:288–295

Asahina K, Zhou B, Pu WT, Tsukamoto H (2011) Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. Hepatology 53:983–995

Basma H, Soto-Gutierrez A, Yannam GR, Liu L, Ito R, Yamamoto T, Ellis E, Carson SD, Sato S, Chen Y, Muirhead D, Navarro-Alvarez N, Wong RJ, Roy-Chowdhury J, Platt JL, Mercer DF,

Miller JD, Strom SC, Kobayashi N, Fox IJ (2009) Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. Gastroenterology 136:990–999

Bisgaard HC, Parmelee DC, Dunsford HA, Sechi S, Thorgeirsson SS (1993) Keratin 14 protein in cultured nonparenchymal rat hepatic epithelial cells: characterization of keratin 14 and keratin 19 as antigens for the commonly used mouse monoclonal antibody OV-6. Mol Carcinog 7:60–66

Bort R, Signore M, Tremblay K, Martinez Barbera JP, Zaret KS (2006) Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. Dev Biol 290:44–56

Cai J, Zhao Y, Liu Y, Ye F, Song Z, Qin H, Meng S, Chen Y, Zhou R, Song X, Guo Y, Ding M, Deng H (2007) Directed differentiation of human embryonic stem cells into functional hepatic cells. Hepatology 45:1229–1239

Carpentier R, Suner RE, van Hul N, Kopp JL, Beaudry JB, Cordi S, Antoniou A, Raynaud P, Lepreux S, Jacquemin P, Leclercq IA, Sander M, Lemaigre FP (2011) Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells. Gastroenterology 141:1432–1438, 1438 e1431–1434

Chen YR, Sekine K, Nakamura K, Yanai H, Tanaka M, Miyajima A (2009) Y-box binding protein-1 down-regulates expression of carbamoyl phosphate synthetase-I by suppressing CCAAT enhancer-binding protein-alpha function in mice. Gastroenterology 137:330–340

Clotman F, Lannoy VJ, Reber M, Cereghini S, Cassiman D, Jacquemin P, Roskams T, Rousseau GG, Lemaigre FP (2002) The onecut transcription factor HNF6 is required for normal development of the biliary tract. Development 129:1819–1828

Clotman F, Jacquemin P, Plumb-Rudewiez N, Pierreux CE, Van der Smissen P, Dietz HC, Courtoy PJ, Rousseau GG, Lemaigre FP (2005) Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. Genes Dev 19:1849–1854

Coffinier C, Gresh L, Fiette L, Tronche F, Schutz G, Babinet C, Pontoglio M, Yaniv M, Barra J (2002) Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. Development 129:1829–1838

Costa RH, Kalinichenko VV, Holterman AX, Wang X (2003) Transcription factors in liver development, differentiation, and regeneration. Hepatology 38:1331–1347

DeLaForest A, Nagaoka M, Si-Tayeb K, Noto FK, Konopka G, Battle MA, Duncan SA (2011) HNF4A is essential for specification of hepatic progenitors from human pluripotent stem cells. Development 138:4143–4153

Deutsch G, Jung J, Zheng M, Lora J, Zaret KS (2001) A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development 128:871–881

Dorrell C, Erker L, Lanxon-Cookson KM, Abraham SL, Victoroff T, Ro S, Canaday PS, Streeter PR, Grompe M (2008) Surface markers for the murine oval cell response. Hepatology 48:1282–1291

Dorrell C, Erker L, Schug J, Kopp JL, Canaday PS, Fox AJ, Smirnova O, Duncan AW, Finegold MJ, Sander M, Kaestner KH, Grompe M (2011) Prospective isolation of a bipotential clonogenic liver progenitor cell in adult mice. Genes Dev 25:1193–1203

Duncan AW, Dorrell C, Grompe M (2009) Stem cells and liver regeneration. Gastroenterology 137:466–481

Dunsford HA, Sell S (1989) Production of monoclonal antibodies to preneoplastic liver cell populations induced by chemical carcinogens in rats and to transplantable Morris hepatomas. Cancer Res 49:4887–4893

Engelhardt NV, Factor VM, Yasova AK, Poltoranina VS, Baranov VN, Lasareva MN (1990) Common antigens of mouse oval and biliary epithelial cells. Expression on newly formed hepatocytes. Differ; Res Biol Div 45:29–37

Farber E (1956) Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylamino-fluorene, and 3′-methyl-4-dimethylaminoazobenzene. Cancer Res 16:142–148

Fausto N (2004) Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. Hepatology 39:1477–1487

Fellous TG, Islam S, Tadrous PJ, Elia G, Kocher HM, Bhattacharya S, Mears L, Turnbull DM, Taylor RW, Greaves LC, Chinnery PF, Taylor G, McDonald SA, Wright NA, Alison MR (2009) Locating the stem cell niche and tracing hepatocyte lineages in human liver. Hepatology 49:1655–1663

Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N, Karasik A (2000) Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nat Med 6:568–572

Floridon C, Jensen CH, Thorsen P, Nielsen O, Sunde L, Westergaard JG, Thomsen SG, Teisner B (2000) Does fetal antigen 1 (FA1) identify cells with regenerative, endocrine and neuroendocrine potentials? A study of FA1 in embryonic, fetal, and placental tissue and in maternal circulation. Differ; Res Biol Div 66:49–59

Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T, Hosokawa S, Elbahrawy A, Soeda T, Koizumi M, Masui T, Kawaguchi M, Takaori K, Doi R, Nishi E, Kakinoki R, Deng JM, Behringer RR, Nakamura T, Uemoto S (2011) Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. Nat Genet 43:34–41

Gai H, Nguyen DM, Moon YJ, Aguila JR, Fink LM, Ward DC, Ma Y (2010) Generation of murine hepatic lineage cells from induced pluripotent stem cells. Differ; Res Biol Div 79:171–181

Geisler F, Nagl F, Mazur PK, Lee M, Zimber-Strobl U, Strobl LJ, Radtke F, Schmid RM, Siveke JT (2008) Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology 48:607–616

Gouon-Evans V, Boussemart L, Gadue P, Nierhoff D, Koehler CI, Kubo A, Shafritz DA, Keller G (2006) BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. Nat Biotechnol 24:1402–1411

Grompe M (2003) Pancreatic-hepatic switches in vivo. Mech Dev 120:99–106

Grozdanov PN, Yovchev MI, Dabeva MD (2006) The oncofetal protein glypican-3 is a novel marker of hepatic progenitor/oval cells. Lab Invest 86:1272–1284

Hirose Y, Itoh T, Miyajima A (2009) Hedgehog signal activation coordinates proliferation and differentiation of fetal liver progenitor cells. Exp Cell Res 315:2648–2657

Hofmann JJ, Zovein AC, Koh H, Radtke F, Weinmaster G, Iruela-Arispe ML (2010) Jagged1 in the portal vein mesenchyme regulates intrahepatic bile duct development: insights into Alagille syndrome. Development 137:4061–4072

Hu M, Kurobe M, Jeong YJ, Fuerer C, Ghole S, Nusse R, Sylvester KG (2007) Wnt/beta-catenin signaling in murine hepatic transit amplifying progenitor cells. Gastroenterology 133:1579–1591

Huang P, He Z, Ji S, Sun H, Xiang D, Liu C, Hu Y, Wang X, Hui L (2011) Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 475:386–389

Itoh T, Kamiya Y, Okabe M, Tanaka M, Miyajima A (2009) Inducible expression of Wnt genes during adult hepatic stem/progenitor cell response. FEBS Lett 583:777–781

Jakubowski A, Ambrose C, Parr M, Lincecum JM, Wang MZ, Zheng TS, Browning B, Michaelson JS, Baetscher M, Wang B, Bissell DM, Burkly LC (2005) TWEAK induces liver progenitor cell proliferation. J Clin Invest 115:2330–2340

Jelnes P, Santoni-Rugiu E, Rasmussen M, Friis SL, Nielsen JH, Tygstrup N, Bisgaard HC (2007) Remarkable heterogeneity displayed by oval cells in rat and mouse models of stem cell-mediated liver regeneration. Hepatology 45:1462–1470

Jensen CH, Jauho EI, Santoni-Rugiu E, Holmskov U, Teisner B, Tygstrup N, Bisgaard HC (2004) Transit-amplifying ductular (oval) cells and their hepatocytic progeny are characterized by a novel and distinctive expression of delta-like protein/preadipocyte factor 1/fetal antigen 1. Am J Pathol 164:1347–1359

Jung J, Zheng M, Goldfarb M, Zaret KS (1999) Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science 284:1998–2003

Kakinuma S, Ohta H, Kamiya A, Yamazaki Y, Oikawa T, Okada K, Nakauchi H (2009) Analyses of cell surface molecules on hepatic stem/progenitor cells in mouse fetal liver. J Hepatol 51:127–138

Kamiya A, Gonzalez FJ (2004) TNF-alpha regulates mouse fetal hepatic maturation induced by oncostatin M and extracellular matrices. Hepatology 40:527–536

Kamiya A, Kinoshita T, Ito Y, Matsui T, Morikawa Y, Senba E, Nakashima K, Taga T, Yoshida K, Kishimoto T, Miyajima A (1999) Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. EMBO J 18:2127–2136

Kamiya A, Kojima N, Kinoshita T, Sakai Y, Miyajima A (2002) Maturation of fetal hepatocytes in vitro by extracellular matrices and oncostatin M: induction of tryptophan oxygenase. Hepatology 35:1351–1359

Kamiya A, Kakinuma S, Yamazaki Y, Nakauchi H (2009) Enrichment and clonal culture of progenitor cells during mouse postnatal liver development in mice. Gastroenterology 137:1114–1126, 1126 e1111–1114

Kim S, Shin JS, Kim HJ, Fisher RC, Lee MJ, Kim CW (2007) Streptozotocin-induced diabetes can be reversed by hepatic oval cell activation through hepatic transdifferentiation and pancreatic islet regeneration. Lab Invest 87:702–712

Kimura T, Christoffels VM, Chowdhury S, Iwase K, Matsuzaki H, Mori M, Lamers WH, Darlington GJ, Takiguchi M (1998) Hypoglycemia-associated hyperammonemia caused by impaired expression of ornithine cycle enzyme genes in C/EBPalpha knockout mice. J Biol Chem 273:27505–27510

Kinoshita T, Sekiguchi T, Xu MJ, Ito Y, Kamiya A, Tsuji K, Nakahata T, Miyajima A (1999) Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. Proc Natl Acad Sci USA 96:7265–7270

Knight B, Akhurst B, Matthews VB, Ruddell RG, Ramm GA, Abraham LJ, Olynyk JK, Yeoh GC (2007) Attenuated liver progenitor (oval) cell and fibrogenic responses to the choline deficient, ethionine supplemented diet in the BALB/c inbred strain of mice. J Hepatol 46:134–141

Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T (2004) The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology 127:1775–1786

Kojima N, Kinoshita T, Kamiya A, Nakamura K, Nakashima K, Taga T, Miyajima A (2000) Cell density-dependent regulation of hepatic development by a gp130-independent pathway. Biochem Biophys Res Commun 277:152–158

Kojima H, Fujimiya M, Matsumura K, Younan P, Imaeda H, Maeda M, Chan L (2003) NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. Nat Med 9:596–603

Kubota H, Reid LM (2000) Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. Proc Natl Acad Sci USA 97:12132–12137

Kyrmizi I, Hatzis P, Katrakili N, Tronche F, Gonzalez FJ, Talianidis I (2006) Plasticity and expanding complexity of the hepatic transcription factor network during liver development. Genes Dev 20:2293–2305

Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, Thorgeirsson SS (2006) A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat Med 12:410–416

Lemaigre FP (2009) Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. Gastroenterology 137:62–79

Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, Qi M, Trask BJ, Kuo WL, Cochran J, Costa T, Pierpont ME, Rand EB, Piccoli DA, Hood L, Spinner NB (1997) Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat Genet 16:243–251

Loomes KM, Russo P, Ryan M, Nelson A, Underkoffler L, Glover C, Fu H, Gridley T, Kaestner KH, Oakey RJ (2007) Bile duct proliferation in liver-specific Jag1 conditional knockout mice: effects of gene dosage. Hepatology 45:323–330

Lozier J, McCright B, Gridley T (2008) Notch signaling regulates bile duct morphogenesis in mice. PLoS One 3:e1851

Ludtke TH, Christoffels VM, Petry M, Kispert A (2009) Tbx3 promotes liver bud expansion during mouse development by suppression of cholangiocyte differentiation. Hepatology 49:969–978

Malato Y, Naqvi S, Schurmann N, Ng R, Wang B, Zape J, Kay MA, Grimm D, Willenbring H (2011) Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. J Clin Invest 121:4850–4860

Margagliotti S, Clotman F, Pierreux CE, Beaudry JB, Jacquemin P, Rousseau GG, Lemaigre FP (2007) The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. Dev Biol 311:579–589

Matsumoto K, Yoshitomi H, Rossant J, Zaret KS (2001) Liver organogenesis promoted by endothelial cells prior to vascular function. Science 294:559–563

Matthews VB, Yeoh GC (2005) Liver stem cells. IUBMB Life 57:549–553

McCright B, Lozier J, Gridley T (2002) A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. Development 129:1075–1082

McDaniell R, Warthen DM, Sanchez-Lara PA, Pai A, Krantz ID, Piccoli DA, Spinner NB (2006) NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. Am J Hum Genet 79:169–173

Michalopoulos GK, DeFrances MC (1997) Liver regeneration. Science 276:60–66

Micsenyi A, Tan X, Sneddon T, Luo JH, Michalopoulos GK, Monga SP (2004) Beta-catenin is temporally regulated during normal liver development. Gastroenterology 126:1134–1146

Newsome PN, Hussain MA, Theise ND (2004) Hepatic oval cells: helping redefine a paradigm in stem cell biology. Curr Top Dev Biol 61:1–28

Nitou M, Sugiyama Y, Ishikawa K, Shiojiri N (2002) Purification of fetal mouse hepatoblasts by magnetic beads coated with monoclonal anti-e-cadherin antibodies and their in vitro culture. Exp Cell Res 279:330–343

Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, Piccoli DA, Meltzer PS, Spinner NB, Collins FS, Chandrasekharappa SC (1997) Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet 16:235–242

Oertel M, Menthena A, Chen YQ, Teisner B, Jensen CH, Shafritz DA (2008) Purification of fetal liver stem/progenitor cells containing all the repopulation potential for normal adult rat liver. Gastroenterology 134:823–832

Oikawa T, Kamiya A, Kakinuma S, Zeniya M, Nishinakamura R, Tajiri H, Nakauchi H (2009) Sall4 regulates cell fate decision in fetal hepatic stem/progenitor cells. Gastroenterology 136:1000–1011

Okabe M, Tsukahara Y, Tanaka M, Suzuki K, Saito S, Kamiya Y, Tsujimura T, Nakamura K, Miyajima A (2009) Potential hepatic stem cells reside in EpCAM+ cells of normal and injured mouse liver. Development 136:1951–1960

Paku S, Schnur J, Nagy P, Thorgeirsson SS (2001) Origin and structural evolution of the early proliferating oval cells in rat liver. Am J Pathol 158:1313–1323

Preisegger KH, Factor VM, Fuchsbichler A, Stumptner C, Denk H, Thorgeirsson SS (1999) Atypical ductular proliferation and its inhibition by transforming growth factor beta1 in the 3,5-diethoxycarbonyl-1,4-dihydrocollidine mouse model for chronic alcoholic liver disease. Lab Invest 79:103–109

Roskams TA, Libbrecht L, Desmet VJ (2003) Progenitor cells in diseased human liver. Semin Liver Dis 23:385–396

Rossi JM, Dunn NR, Hogan BL, Zaret KS (2001) Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev 15:1998–2009

Rountree CB, Barsky L, Ge S, Zhu J, Senadheera S, Crooks GM (2007) A CD133-expressing murine liver oval cell population with bilineage potential. Stem Cells 25:2419–2429

Sackett SD, Li Z, Hurtt R, Gao Y, Wells RG, Brondell K, Kaestner KH, Greenbaum LE (2009) Foxl1 is a marker of bipotential hepatic progenitor cells in mice. Hepatology 49:920–929

Schmelzer E, Zhang L, Bruce A, Wauthier E, Ludlow J, Yao HL, Moss N, Melhem A, McClelland R, Turner W, Kulik M, Sherwood S, Tallheden T, Cheng N, Furth ME, Reid LM (2007) Human hepatic stem cells from fetal and postnatal donors. J Exp Med 204:1973–1987

Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, Birchmeier C (1995) Scatter factor/hepatocyte growth factor is essential for liver development. Nature 373:699–702

Schrem H, Klempnauer J, Borlak J (2002) Liver-enriched transcription factors in liver function and development. Part I: the hepatocyte nuclear factor network and liver-specific gene expression. Pharmacol Rev 54:129–158

Schrem H, Klempnauer J, Borlak J (2004) Liver-enriched transcription factors in liver function and development. Part II: the C/EBPs and D site-binding protein in cell cycle control, carcinogenesis, circadian gene regulation, liver regeneration, apoptosis, and liver-specific gene regulation. Pharmacol Rev 56:291–330

Sekine K, Chen YR, Kojima N, Ogata K, Fukamizu A, Miyajima A (2007) Foxo1 links insulin signaling to C/EBPalpha and regulates gluconeogenesis during liver development. EMBO J 26:3607–3615

Sekiya S, Suzuki A (2011) Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 475:390–393

Shin S, Walton G, Aoki R, Brondell K, Schug J, Fox A, Smirnova O, Dorrell C, Erker L, Chu AS, Wells RG, Grompe M, Greenbaum LE, Kaestner KH (2011) Foxl1-Cre-marked adult hepatic progenitors have clonogenic and bilineage differentiation potential. Genes Dev 25:1185–1192

Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA (2010) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51:297–305

Snykers S, De Kock J, Rogiers V, Vanhaecke T (2009) In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. Stem Cells 27:577–605

Song YD, Lee EJ, Yashar P, Pfaff LE, Kim SY, Jameson JL (2007) Islet cell differentiation in liver by combinatorial expression of transcription factors neurogenin-3, BETA2, and RIPE3b1. Biochem Biophys Res Commun 354:334–339

Song Z, Cai J, Liu Y, Zhao D, Yong J, Duo S, Song X, Guo Y, Zhao Y, Qin H, Yin X, Wu C, Che J, Lu S, Ding M, Deng H (2009) Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. Cell Res 19:1233–1242

Sosa-Pineda B, Wigle JT, Oliver G (2000) Hepatocyte migration during liver development requires Prox1. Nat Genet 25:254–255

Strick-Marchand H, Masse GX, Weiss MC, Di Santo JP (2008) Lymphocytes support oval cell-dependent liver regeneration. J Immunol 181:2764–2771

Sullivan GJ, Hay DC, Park IH, Fletcher J, Hannoun Z, Payne CM, Dalgetty D, Black JR, Ross JA, Samuel K, Wang G, Daley GQ, Lee JH, Church GM, Forbes SJ, Iredale JP, Wilmut I (2010) Generation of functional human hepatic endoderm from human induced pluripotent stem cells. Hepatology 51:329–335

Suzuki A, Zheng Y, Kondo R, Kusakabe M, Takada Y, Fukao K, Nakauchi H, Taniguchi H (2000) Flow-cytometric separation and enrichment of hepatic progenitor cells in the developing mouse liver. Hepatology 32:1230–1239

Suzuki A, Zheng YW, Kaneko S, Onodera M, Fukao K, Nakauchi H, Taniguchi H (2002) Clonal identification and characterization of self-renewing pluripotent stem cells in the developing liver. J Cell Biol 156:173–184

Suzuki A, Iwama A, Miyashita H, Nakauchi H, Taniguchi H (2003) Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells. Development 130:2513–2524

Suzuki T, Kanai Y, Hara T, Sasaki J, Sasaki T, Kohara M, Maehama T, Taya C, Shitara H, Yonekawa H, Frohman MA, Yokozeki T, Kanaho Y (2006) Crucial role of the small GTPase ARF6 in hepatic cord formation during liver development. Mol Cell Biol 26:6149–6156

Suzuki A, Sekiya S, Buscher D, Izpisua Belmonte JC, Taniguchi H (2008a) Tbx3 controls the fate of hepatic progenitor cells in liver development by suppressing p19ARF expression. Development 135:1589–1595

Suzuki A, Sekiya S, Onishi M, Oshima N, Kiyonari H, Nakauchi H, Taniguchi H (2008b) Flow cytometric isolation and clonal identification of self-renewing bipotent hepatic progenitor cells in adult mouse liver. Hepatology 48:1964–1978

Suzuki K, Tanaka M, Watanabe N, Saito S, Nonaka H, Miyajima A (2008c) p75 Neurotrophin receptor is a marker for precursors of stellate cells and portal fibroblasts in mouse fetal liver. Gastroenterology 135(270–281):e273

Tanaka M, Hirabayashi Y, Sekiguchi T, Inoue T, Katsuki M, Miyajima A (2003) Targeted disruption of oncostatin M receptor results in altered hematopoiesis. Blood 102:3154–3162

Tanaka M, Okabe M, Suzuki K, Kamiya Y, Tsukahara Y, Saito S, Miyajima A (2009) Mouse hepatoblasts at distinct developmental stages are characterized by expression of EpCAM and DLK1: drastic change of EpCAM expression during liver development. Mech Dev 126:665–676

Tanaka M, Itoh T, Tanimizu N, Miyajima A (2011) Liver stem/progenitor cells: their characteristics and regulatory mechanisms. J Biochem 149:231–239

Tang DQ, Lu S, Sun YP, Rodrigues E, Chou W, Yang C, Cao LZ, Chang LJ, Yang LJ (2006) Reprogramming liver-stem WB cells into functional insulin-producing cells by persistent expression of Pdx1- and Pdx1-VP16 mediated by lentiviral vectors. Lab Invest 86:83–93

Tanimizu N, Miyajima A (2004) Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. J Cell Sci 117:3165–3174

Tanimizu N, Miyajima A (2007) Molecular mechanism of liver development and regeneration. Int Rev Cytol 259:1–48

Tanimizu N, Tsujimura T, Takahide K, Kodama T, Nakamura K, Miyajima A (2004) Expression of Dlk/Pref-1 defines a subpopulation in the oval cell compartment of rat liver. Gene Expr Patterns 5:209–218

Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR, Darlington GJ (1995) Impaired energy homeostasis in C/EBP alpha knockout mice. Science 269:1108–1112

Wang X, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M (2003) The origin and liver repopulating capacity of murine oval cells. Proc Natl Acad Sci USA 100(Suppl 1):11881–11888

Wang AY, Ehrhardt A, Xu H, Kay MA (2007) Adenovirus transduction is required for the correction of diabetes using Pdx-1 or Neurogenin-3 in the liver. Mol Ther 15:255–263

Watanabe T, Nakagawa K, Ohata S, Kitagawa D, Nishitai G, Seo J, Tanemura S, Shimizu N, Kishimoto H, Wada T, Aoki J, Arai H, Iwatsubo T, Mochita M, Watanabe T, Satake M, Ito Y, Matsuyama T, Mak TW, Penninger JM, Nishina H, Katada T (2002) SEK1/MKK4-mediated SAPK/JNK signaling participates in embryonic hepatoblast proliferation via a pathway different from NF-kappaB-induced anti-apoptosis. Dev Biol 250:332–347

Weinstein M, Monga SP, Liu Y, Brodie SG, Tang Y, Li C, Mishra L, Deng CX (2001) Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on beta1-integrin to promote normal liver development. Mol Cell Biol 21:5122–5131

Yang L, Li S, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peck AB (2002) In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci USA 99:8078–8083

Yang W, Yan HX, Chen L, Liu Q, He YQ, Yu LX, Zhang SH, Huang DD, Tang L, Kong XN, Chen C, Liu SQ, Wu MC, Wang HY (2008) Wnt/beta-catenin signaling contributes to activation of normal and tumorigenic liver progenitor cells. Cancer Res 68:4287–4295

Yechoor V, Liu V, Espiritu C, Paul A, Oka K, Kojima H, Chan L (2009) Neurogenin3 is sufficient for transdetermination of hepatic progenitor cells into neo-islets in vivo but not transdifferentiation of hepatocytes. Dev Cell 16:358–373

Yovchev MI, Grozdanov PN, Joseph B, Gupta S, Dabeva MD (2007) Novel hepatic progenitor cell surface markers in the adult rat liver. Hepatology 45:139–149

Yovchev MI, Zhang J, Neufeld DS, Grozdanov PN, Dabeva MD (2009) Thymus cell antigen-1-expressing cells in the oval cell compartment. Hepatology 50:601–611

Zajicek G, Oren R, Weinreb M Jr (1985) The streaming liver. Liver 5:293–300

Zalzman M, Gupta S, Giri RK, Berkovich I, Sappal BS, Karnieli O, Zern MA, Fleischer N, Efrat S (2003) Reversal of hyperglycemia in mice by using human expandable insulin-producing cells differentiated from fetal liver progenitor cells. Proc Natl Acad Sci USA 100:7253–7258

Zhao R, Duncan SA (2005) Embryonic development of the liver. Hepatology 41:956–967

Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F, Stanger BZ (2009) Notch signaling controls liver development by regulating biliary differentiation. Development 136:1727–1739

Chapter 14
Gastro Intestinal Stem Cells

M. Sasikala, G.V. Rao, Manu Tandan, and D. Nageshwar Reddy

Abstract Gastro intestinal epithelium (GI) is a rapidly proliferating tissue and is a suitable model for study of stem cell biology. GI stem cells are mainly identified as esophageal, gastric, intestinal, hepatic and pancreatic stem cells. Intestinal stem cells are most extensively studied in mouse models. In the small intestine stem cells reside at the base of the crypt at the 4th position and give rise to all cell lineages of the crypt, while in the large intestine stem cells are located in the mid crypt region and do not undergo apoptosis. Gastric stem cells in the pyloric antrum give rise to pit cell, parietal cell and zymogenic cell lineages and those in the small and large intestine differentiate into absorptive goblet, paneth, columnar cells, and deep cell secretory cell lineages. Wnt signaling plays a major role controlling cell proliferation, differentiation and apoptosis in the crypt-villus axis along with coordinated notch signaling in the gastro intestinal tract. The importance of cancer stem cells is being evaluated in colorectal cancers in the recent years. Understanding the basic mechanisms of differentiation of GI stem cells and establishing in vitro models systems to culture and propogate the GI stem cells is of utmost importance before they are put to therapeutic use in clinical applications.

M. Sasikala, Ph.D., MAMS (✉)
Institute of Basic Sciences and Translational Research, Asian Healthcare Foundation, 6-3-661, Somajiguda, Hyderabad 500082, India
e-mail: aigres.mit@gmail.com

G.V. Rao, M.S., MAMS, FRCS
Surgical Gastroenterology, Medical Gastroenterology Asian Institute of Gastroenterology, 6-3-661, Somajiguda, Hyderabad 500082, India

M. Tandan, M.D., D.M.
D. Nageshwar Reddy, M.D., D.M., D.Sc, FAMS FRCP
Medical Gastroenterology, Asian Institute of Gastroenterology, 6-3-661, Somajiguda, Hyderabad 500082, India

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_14, © Springer Science+Business Media Dordrecht 2013

Abbreviations

GI	Gastro intestinal.
GIT	Gastro intestinal tract.
GLS	Gastric luminal surface.
GTP	Guanosine-5′-triphosphate.
DCS	Deep crypt secretory cell
TGFβ	Transforming growth factor *beta.*
TCF4	Transcription factor 4.
BMP	Bone morphogenetic proteins.
SHH	*Sonic hedgehog homolog.*
IHH	Indian hedgehog.
DHH	Desert hedgehog.
SFRP1	*Secreted frizzled-related protein 1.*
CSCs	Cancer Stem Cells.
ESA	Epithelial-specific antigen.
HCC	Hepatocellular carcinoma.
Mtor	Mammalian target of rapamycin.
HSCT	Hematopoietic stem cells transplantation.
MSCs	Mesenchymal stem cells.
BM-SC	Bone marrow derived stem cells.
G-CSF	Granulocyte colony-stimulating factor.

14.1 Introduction

The identification of undifferentiated cells (stem cells) among differentiated cells in adult tissues offers enormous scope for regenerative medicine. In adults, stem cells and progenitor cells act as repair systems to replenish cell loss that occurs due to injury, damage or disease. Stem cells are known to reside in specific areas (niches) within different tissues including the gastrointestinal tract. Since the use of adult stem cells is not controversial from an ethical perspective, concerned research has generated a lot of excitement among the scientific community. The present review attempts to summarize recent knowledge on gastro intestinal stem cells since the gastrointestinal tract represents the largest reservoir of stem cells and offers significant opportunities in regenerative medicine as also in understanding development, homeostasis and carcinogenesis.

14.1.1 Gastrointestinal Epithelium

Gastro intestinal tract (GIT) is lined by single layer of epithelial cells representing a dynamic barrier for the facilitated entry of food materials and drugs. GIT experiences continuous cell loss through high rates of mechanical attrition. The intestinal epithelium

has to be continually replenished by striking a balance between epithelial cell apoptosis and regeneration in order to maintain structural integrity which is essential for maintaining cellular homeostasis and functional efficiency. Unlike gastric glands made of parietal cells, chief cells, mucus-secreting cells and hormone-secreting cells (endocrine cells), the intestinal epithelium is a simple columnar epithelial lining made of crypts and villi. The villus is composed of three main differentiated cell types namely absorptive enterocyte lineages, mucus secreting goblet cells, Paneth cells and entero endocrine cells. The maintenance of the intestinal epithelium is secured by rapid renewal in 3–5 days (Moore and Lemischka 2006), by local stem cells, resulting in near complete tissue turnover approximately every 7 days. This robust and dynamic process is tightly regulated by a small group of intestinal stem cells residing in the crypts of the intestinal villi. Because, these stem cells are responsible for the continual generation of all intestinal epithelial lineages (secretory goblet, and the entero endocrine cells), the intestine thus serves as one of the most powerful systems for the study of stem cell biology.

14.1.2 Stem Cells in Gastro Intestinal Tract

The presence of adult stem cell like cells in gastrointestinal (GI) tract was postulated 60 years ago (Leblond et al. 1948) and were identified mainly as intestinal, esophageal, hepatic and pancreatic stem cells (Quante and Wang 2009). Gastro intestinal stem cells can be considered as mutipotent cells capable of self renewal because of their ability to differentiate into different kinds of cells characteristic of the gastric and intestinal epithelium. These GI stem cells could provide promising sources for cellular therapies in the treatment of gastrointestinal diseases. Progress in the GI stem cell biology could be achieved with the advent of putative stem cell markers that distinguished quiescent and active stem cell populations. While base line regeneration of intestinal stem cells is accomplished by active stem cell population, quiescent cells respond to injury (Li and Clevers 2010; Scoville et al. 2008).

14.2 Classification and Derivation of Gastro Intestinal Stem Cells

Based on the location of stem cells in the GI tract they can be classified mainly into gastric and intestinal stem cells.

14.2.1 Classification

Gastric stem cells: It was demonstrated that oxyntic epithelium of the gastric glands contains the progenitors in the isthmus region of the pit gland and the cells lining the pit gland unit of the oxyntic epithelium originating from stem cells give rise to the three main cell lineages of the stomach (Karam and Leblond 1993a).

Intestinal stem cells: The functional unit of small intestine is the crypt-villus axis and production and delivery of cells to the villus is the primary function of crypt (Bjerknes and Cheng 2005). Stem cells in the small intestine were found to be situated above the paneth cell in the crypt forming the stem cell niche in the intestine (Potten et al. 1997, 2002; Batlle 2008). It is now confirmed and precisely defined in the 4th cell at the bottom of the crypt (Potten et al. 2002).

Hepatic and Pancreatic stem cells are not in the purview of this review.

14.2.2 Derivation of Stem Cells

GI tract is endodermal in origin and is a single layer of proliferative endodermal cells which increases its surface area by production of various cell lineages of gastrointestinal system (Maunoury et al. 1992).

In general, mature GI epithelial cells are formed through five main stages as shown below.

General schematic representation of Gastro intestinal stem cell differentiation

Stem Cells
(stage1)
↓
Uncommitted precursor cells
(stage2: exhibit dual lineage features)
(Lee and Leebond, Karam and Leblond)
↓
Committed precursor cells
(stage3: Transit cells)
↓
Mature cells
(stage4: differentiated functional cells)
↓
Terminal cells
(Karam et al.)
(Stage5: death and elimination)

14.3 Characteristics of Gastrointestinal Stem Cells

Intestinal stem cells have been extensively studied in mouse models. In small intestine the stem cells are demonstrated to be located at about the fourth position from the bottom of the crypt and in large intestine (colon) they are located at the base of the crypt. Stem cells in the crypt are organized in three different tiers (Potten and Hendry 1995). At the base of the hierarchy are the ancestral stem cells; four to six

in number. When they undergo apoptosis, second tier of stem cells, six in number take over the function of ancestral cells. A third tier of 24 stem cells with greater resistance and repair strength regenerate the earlier tiers of stem cells. Therefore there are about 36 stem cells in the crypt. Cell proliferation studies and mathematical modeling studies indicated that the each crypt containing 250 cells in a flask shaped structure with 150–160 rapidly proliferating cells and 30 differentiated functional paneth cells is maintained by the three tiers of stem cells at the base of the crypt. Most of these cells divide once a day at the base of the crypt. It was noted that some cells at the base specifically involved in regeneration after injury undergo low level of spontaneous apoptosis and represent the origin of migration (Potten 1998). Approximately 1–10% of the cells at stem cell position undergo apoptosis at any time in small intestine where as spontaneous apoptosis is rare in large intestine. Spontaneous apoptosis is induced in GI stem cell population in about 3–6 h by chemical mutagens and cytotoxic drugs which is totally p53 independent (Merritt et al. 1977).

14.4 Differentiation of Stem Cells

14.4.1 Gastric Epithelial Cell Lineage

The cells lining the pit gland unit differentiate into gastric epithelial cells and have a turn over time of 2.5 days (Karam and Leblond 1993a). Stem cells in the body of the stomach differentiate into Pit cell lineage, parietal cell lineage and zymogen cell lineage as shown in Fig. 14.1. It was shown that duration of cell cycle in pyloric antrum is about 8.4 h (El-Alfy and Leblond 1987a).

14.4.1.1 Pit Cell Lineage

About 67% of the progeny of the stem cells in the isthmus region become pre pit cell precursors that are characterized by small Golgi apparatus and are partially committed. About 99% of the progeny cells become pre-pit cells and 1% becomes pre-parietal cells by maturation of Golgi vesicles into dense secretory granules (Karam and Leblond 1993a). Pre pit cells (~10 in each isthmus) are located in the upper region containing 200 nm wide secretory granules. Fifty seven percent of these cells arise from pre-pit cell precursors while the remaining 43% arise from their own mitosis. The activity of pre-pit cells increases and increasing number of secretory granules are produced at the apex to become pit cells. The pit cells thus formed are characterized by dense apical group of mucus granules and migrate to reach gastric luminal surface (Karam and Leblond 1993b). The time taken for pre pit cell to become pit cell is 3 days (Karam and Leblond 1993b).

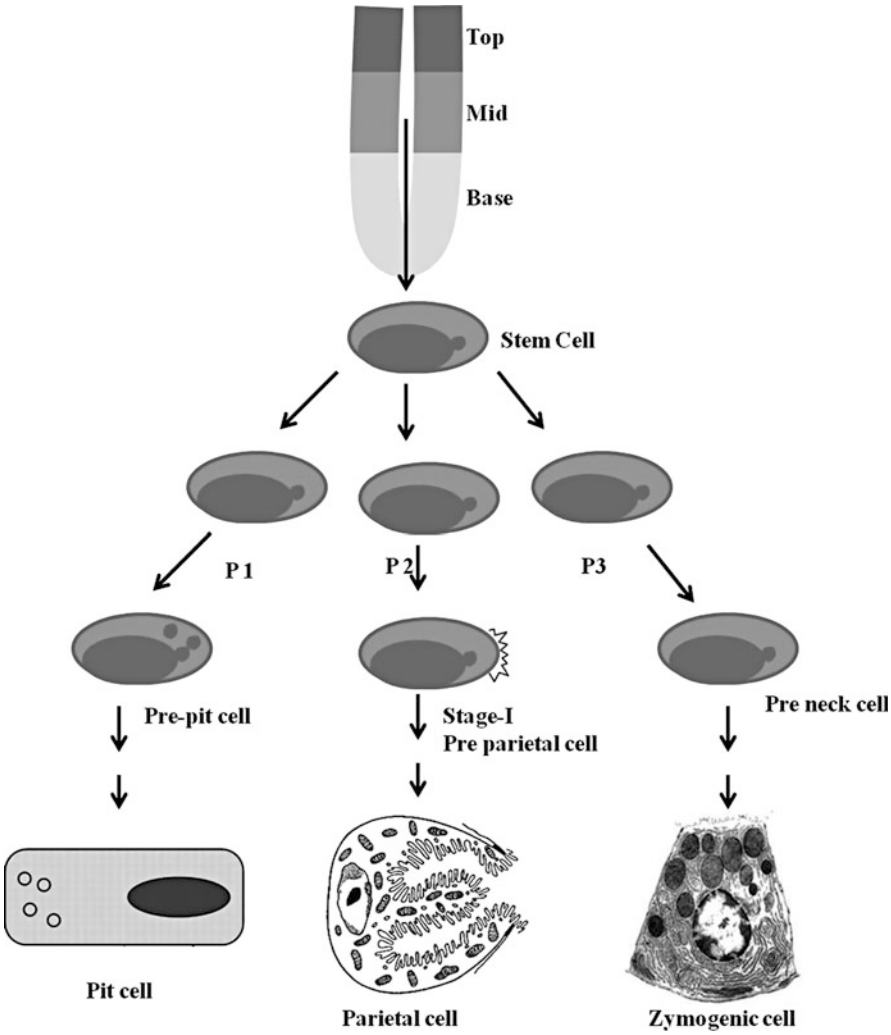


Fig. 14.1 Pit gland unit of stomach. Stem cells in the isthmus region of the gastric epithelium initially differentiate into precursor cells and later evolve into mature pit cells, parietal cells and zymogenic cells

14.4.1.2 Parietal Cell Lineage

Pre-parietal cell precursors that arise from the stem cells in GI epithelium are characterized by embryonic cell like features in addition to numerous apical villi (Li et al. 1995) and differentiate to form pre-parietal cells. These precursors are divided into three groups based on the presence or absence of secretory granules; these are pre-parietal cells with no secretory granules, those with small dense gran-

ules and those with cored granules. Pre-parietal cells differentiate into parietal cells in four stages characterized by increase in the surface area of apical plasma membrane, appearance of tubules and vesicles, increase in the number of mitochondria, and overall increase in cell size leading to formation of fully mature parietal cell (Karam and Leblond 1993a). Maturation of pre parietal cell takes about 1 day while that of parietal cell takes 2 days (Karam et al. 1997). Autoradiography studies revealed that they migrate bi directionally along the pit gland axis and their turnaround time is around 54 days. Parietal cells were shown to produce regulatory factors required for terminal differentiation of zymogenic cells (Mills et al. 2001).

14.4.1.3 Zymogenic Cell Lineage

Stem cells that are committed to zymogenic cell lineage (24%) become pre neck cells. These precursors are characterized by pro secretory granules, 98% of which becomes pre neck cells and 2% becomes pre parietal cells. Pre neck cells are located in the lower portion of isthmus (1.8 cells/isthmus with 400 nm wide secretory granules) and develop into neck cells in a turnaround time of 3 days (Karam and Leblond 1993a) and transform into neck cells located in the neck region characterized by dense mucus granules (Sato and Spicer 1980). Such transformed cells migrate while completing their differentiation into mucus producing cells. After 7–14 days in the neck, they slowly get converted to serous cells. Cells at the base region of the pit gland are the pre zymogenic cells that produce secretory granules which contain mucus and pepsinogenic granules. These cells gradually transform into pepsinogenic granule containing cells rather than dense mucus containing cells. These zymogenic cells are pepsinogen secreting cells with 700–1,070 nm wide secretory granules and a turn over time of 194 days (Karam and Leblond 1993c) and degenerate at the bottom of the pit gland. The cell lineages in the pyloric antrum are almost similar to those in the body of the stomach except immediate descendents are called as mottled granular cells with embryonic cell like features (Lee and Leblond 1985a). These precursor cells undergo clonal expansion and become pre pit cells and pre gland cells. Transformation of pre pit cells is similar to that of oxyntic epithelium (Lee 1985). Pre gland cells arising from the stem cells are poorly differentiated cells representing 28%of the isthmus cells, duplicate, migrate and differentiate to form gland cells (Lee and Leblond 1985b).

14.4.2 Small Intestinal Cell Lineage

Small intestinal epithelial stem cells have been extensively studied in mice models. Epithelium in the small intestine undergoes invagination to form small crypts and evagination to form large villi. The crypts contain both immediate precursors and proliferative stem cells (Potten and Loeffler 1990) that give rise to GI epithelial

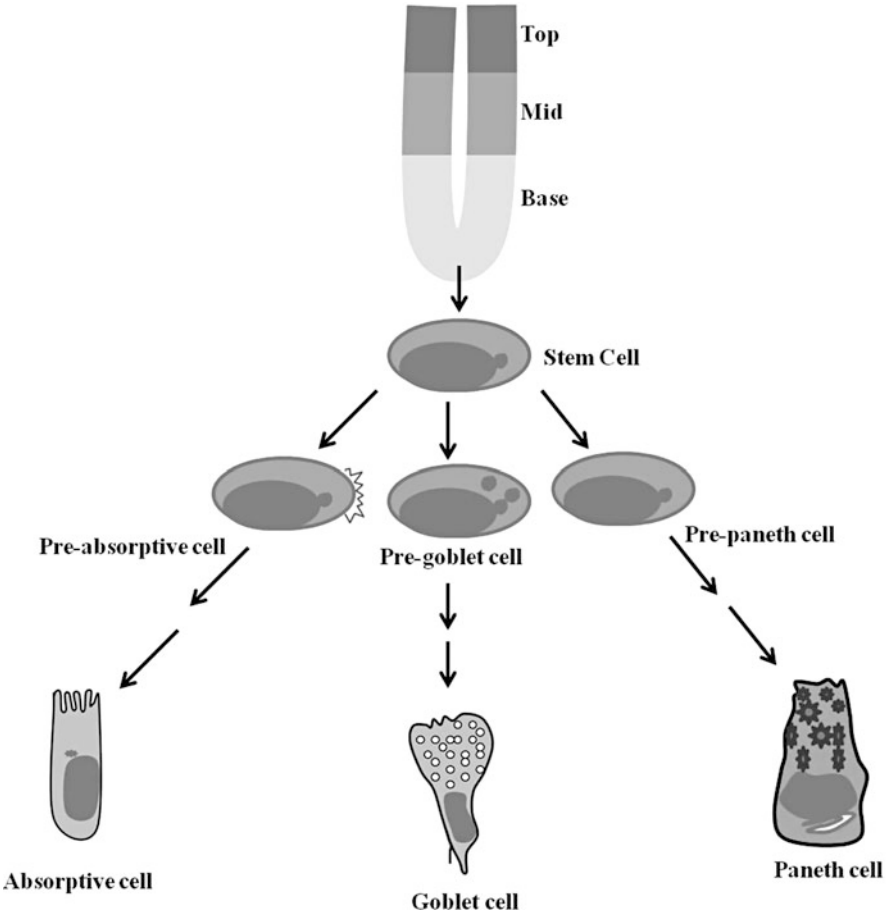


Fig. 14.2 Intestinal crypt cell differentiation. Intestinal crypt cell located at the base (4th position) differentiates into precursor cells which later give rise to absorptive cell, goblet cell and paneth cell lineages

cells. It is estimated that each crypt contains 250 cells (Li et al. 1994) and 2/3 of these cells undergo cell cycle every 12 h producing 13–16 cells/h which differentiate to give rise to cells of absorptive cell lineage, goblet cell lineage, and paneth cell lineage (Potten et al. 1997) (Fig. 14.2).

14.4.2.1 Absorptive Cell Lineage

Pre-Absorptive cells: Stem cells at the base of the crypts proliferate to become pre absorptive cells which possess long microvilli with stem cell like features and are capable of mitotic division. These cells migrate to the middle of the crypt and gradually

differentiate into brush border cells with elongated micro villi. Absorptive cells are completely differentiated from pre absorptive cells by the time they reach the top of the crypt. These absorptive cells are located at the top of the crypt and all along the villus are characterized by absence of secretory granules and prominent apical brush border with an average turnover time of 3 days (Cheng 1974a). Certain adhesion molecules (Troughton and Trier 1969), extra cellular matrix proteins (Lee and Leblond 1985a, b) and Rac1 (member of family of GTP proteins) play an important role in the maintenance, differentiation and homeostasis of intestinal epithelium.

14.4.2.2 Goblet Cell Lineage

Pre-Goblet cells: Pre-goblet cells are found at the base of the crypt. They have stem cells features with limited mitotic capacity. Appearance of few small mucous granules within these cells denotes the sign of early differentiation of these cell types. Pre-goblet cells originate by their own mitosis and differentiation of stem cells. The outward migration of these cells towards mid crypt region in 12–24 h is characterized by accumulation of more supra nuclear cytoplasmic granules leading to the transformation of these cells to functional goblet cells (Cheng 1974a). The turnover time of goblet cells is about 3 days. Such differentiated goblet cells migrate to the tip of the villus and are characterized by large group of mucus granules.

14.4.2.3 Paneth Cell Lineage

The number, morphology and presence of the paneth cells vary across different species. They are present at the lower crypt region and constitute 3.3–7.5% of dueodenal, jejunal and ileal epithelium and arise from the pre paneth cells.

Pre-Paneth cells: These cells having stem cell like features are located at the base of the crypt adjacent to their ancestor stem cells. Upon migration towards the bottom of the crypt, they differentiate to generate mature functional paneth cells (Cheng and Leblond 1974b). Paneth cells are differentiated only from the stem cells and do not have mitotic activity (Troughton and Trier 1969). They are characterized by secretory granules measuring 500 nm in the mid crypt and 3,000 nm in the bottom of the crypt. Turn over time of paneth cells is about 15 days and they migrate to the bottom of the crypt.

Cell lineages in colon: The ascending and descending portions of the colon show variations in the size of crypts, type of lining cells and dynamicity of the colonic stem cells (Chang and Nadler 1975). In the ascending colon, stem cells are located in the mid crypt region migrating bidirectionally, while they are located at the base of the crypt in the descending colon with outward migration (Sato and Ahnen 1992). More number of cells undergoes proliferation for a longer period in the ascending colon as compared to descending colon. which explains higher risk of colon cancer in the descending colon.

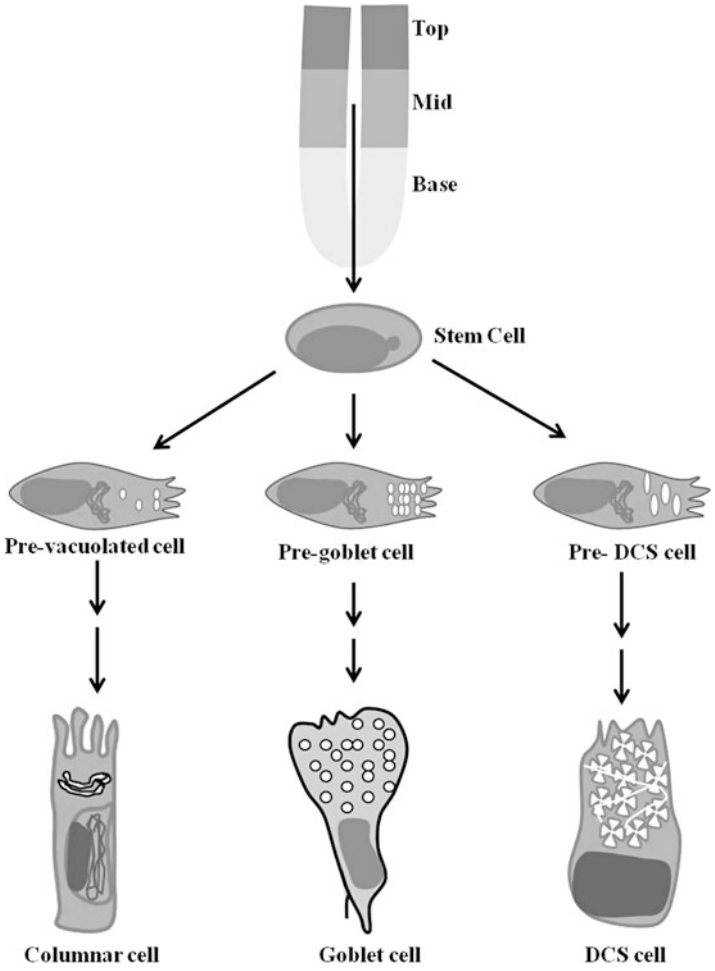


Fig. 14.3 Differentiation of crypt cells in ascending colon. Stem cells in the mid crypt region of ascending colon differentiate into precursors which mature to give rise to columnar cell, goblet cell and deep crypt secretory cell lineages

Colonic stem cells undergo differentiation to give rise to vacuolated columnar cell lineage, goblet cell lineage and deep crypt secretory (DCS) cell lineage (Fig. 14.3).

14.4.3 Ascending and Descending Colon Cell Lineage

These cells are located in the mid crypt region. They have an average cell cycle time of 19 h and the average number of proliferating cells are 90/crypt. A bidirectional

mode of migration is observed in the stem cells of ascending colon. Contrary to the ascending colon, stem cells of descending colon are localized at the base of the crypt. The average cell cycle time of these cells is 15 h (shorter than ascending colon) and the average number of proliferating cells are 190/crypt far exceeding the numbers in ascending colon. Due to the presence of much higher number of proliferating cells, risk of colon cancer is much higher in the descending colon. Furthermore, in comparison to the cells of ascending colon, migration of stem cells of descending colon occurs in an outward direction (Sato and Ahnen 1992).

14.4.3.1 Columnar Cell Lineage

Majority of the crypt base (80%) is constituted by vacuolated columnar cell lineage comprising of three types of cells namely pre vacuolated cells, vacuolated cells and columnar cells. Pre vacuolated cells develop from maturation of stem cells and accumulate granules to become vacuolated cells (Chang and Leblond 1971). These are located upper and lower two thirds of crypts of ascending and descending colon. Gradually the vacuolated cells acquire striated border and differentiate into absorptive columnar epithelium which takes about 3 days (Chang and Nadler 1975). Columnar cells are found in the crypt top of both ascending and descending colon, and are less prominent than absorptive cells of small intestine.

14.4.3.2 Goblet Cell Lineage

Goblet cell lineage is characterized by mucous granules corresponding to 16% of crypt cell population (Chang and Leblond 1971). Pre goblet cells are developed from maturation of stem cells and by their own mitosis. After they are formed, pre goblet cells move upward the crypt and acquire more number of granules to transform into goblet cells in about 2 days (Chang and Nadler 1975). Goblet cells are characterized by numerous large mucous granules.

14.4.3.3 Deep Crypt Secretory Cell Lineage

These cells are produced from stem cells located in mid crypt as pre deep crypt secretory cells. They differ from goblet cells in their ultra structure (Altmann 1983) and undergo differentiation and maturation as they migrate to the bottom of the crypt which takes 14–21 days (Altmann 1990).

14.4.4 Stem Cells Scattered Along GI Tract

The enteroendocrine cells that produce peptide or polypeptides are scattered in the gut epithelium throughout the intestinal tract. This cell types share a common stem cell origin with other GI stem cells (Karam and Leblond 1993d).

Pre-Enteroendocrine Cells: These are immature cells that originate mainly by self mitosis as well as by differentiation of stem cells and contain very few endocrine-like secretory granules. They are located in the isthmus region of the gastric oxyntic glands (Karam and Leblond 1993d) and the base of the crypt in the epithelium of small intestine (Cheng and Leblond 1974b) and descending colon (Tsubouchi and Leblond 1979). Differentiation and maturation of these cells occur during their migration and they are finally transformed into mature enteroendocrine cells with the ability to produce more granules.

14.4.5 Stem Cells Associated to Lymphoid Follicles

These cells lack mitotic ability and originate from the stem cells located at the base of crypt in the small and large intestine. Upon generation, these cells undergo outward migration, covering the lymphoid follicles as differentiated and matured antigen-sampling M cells (Gebert and Posselt 1997).

14.5 Cell Signaling in Stem Cell Niche

The uniqueness of GI epithelium lies in the orderly maneuvering of cell proliferation, differentiation, and apoptosis along the crypt-villus axis. The intestinal crypt is maintained by stem cells and mainly acts as a proliferative compartment while the villus receives cells from multiple crypts to act as a differentiated compartment. In other words, intestinal crypt is monoclonal while the villus is polyclonal in nature. To maintain the integrity of the intestinal epithelium, stem cells require a protective niche to carry out their self renewal, proliferation and differentiation activities. This environment is maintained by the epithelial- mesenchymal crosstalk where proliferating and differentiating epithelial cells are surrounded by mesenchymal cells.

Since the GI tract is made up of an endodermally-derived epithelium surrounded by cells of mesoderm origin, cell signaling between these two tissue layers plays a critical role in coordinating the patterning and organogenesis of the gut and its derivatives. Over the years, although Wnt signaling appeared as a major force in controlling cell proliferation, differentiation and apoptosis along the crypt-villus axis, other factors like TGF-beta, homeobox, forkhead, hedgehog, homeodomain, and platelet-derived growth factor are also gaining importance contributing to stem cell signaling in the gastrointestinal tract (Yen and Wright 2006).

Wnt signaling in GI stem cells: The highly conserved Wnt family of secreted molecules constitutes Wnt signaling which is activated in the progenitor (transit-amplifying) region around the bottom of intestinal crypt and plays a key regulatory role for the maintenance of intestinal epithelium. Earlier studies indicate that stem cell proliferation in GI tract requires Wnt signaling but their survival and maintenance in the stem cell state, at least for short term seem to be Wnt independent (Pinto et al. 2003). Gene expression profiling of Wnt signaling pathway in the human colonic crypt demonstrated differential

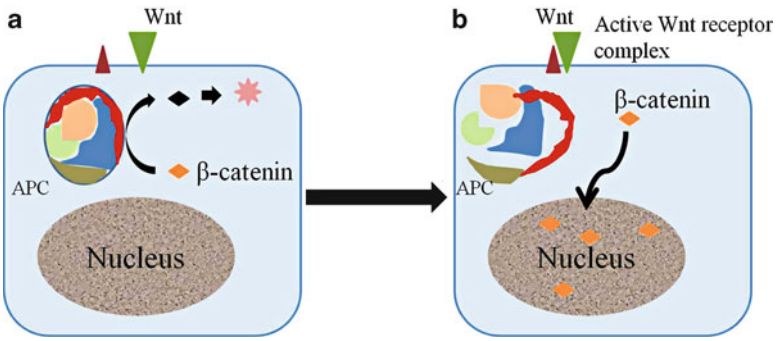


Fig. 14.4 Wnt signaling. Binding of Wnt to its receptor results in activation of β catenin which enters the nucleus and combines with TCF/LEF transcription factors and induces transcription of genes involved in proliferation. APC protein complex inhibits β catenin activation causing its internalization and degradation

expression of Wnt signaling molecules (Kosinki et al. 2007) (Fig. 14.4). Studies involving deletion of TCF4 (an end target of Wnt signaling), (Spradling et al. 2001) and/or a mutation in the β catenin were shown to result in reduced proliferative capabilities of gastro intestinal stem cells and loss of entire crypts (Sancho et al. 2003). While Wnt and Notch signaling were shown to coordinate GI epithelial cell homeostasis as shown in the Fig. 14.5, Bmp signaling generated in the mesenchyme in turn was shown to inhibit stem cell self renewal through modulation of Wnt activity (He et al. 2004).

14.5.1 The TGF-β Signaling in GI Stem Cells

The TGF-β superfamily signals are conveyed through serine/threonine kinase receptors to specific intracellular mediators known as the Smad proteins. It has been implicated that TGF-β pathway has a prominent role in GI progenitor cell formation and differentiation, disruptions of which leads to cancer. Studies have localized TGF-β type II receptors to both differentiated epithelial cells of the villi as well as undifferentiated crypt cells. It is likely that TGF-β signaling is important in transitioning of a stem cell into a proliferative progenitor phenotype in the crypts where as TGF-β signaling may be required for apoptosis at the villus tips to maintain the normal size, shape, and function of the polarized gut epithelium (Mishra et al. 2005).

14.5.2 Hedgehog Signaling in GI Stem Cells

Hedgehog signaling, which occur in the differentiated region around the surface of intestinal villi, plays a key role in embryogenesis, maintenance of adult tissue homeostasis, tissue repair during chronic persistent inflammation, and carcinogenesis. Hedgehog family ligands (Sonic hedgehog [SHH], Indian hedgehog [IHH] and

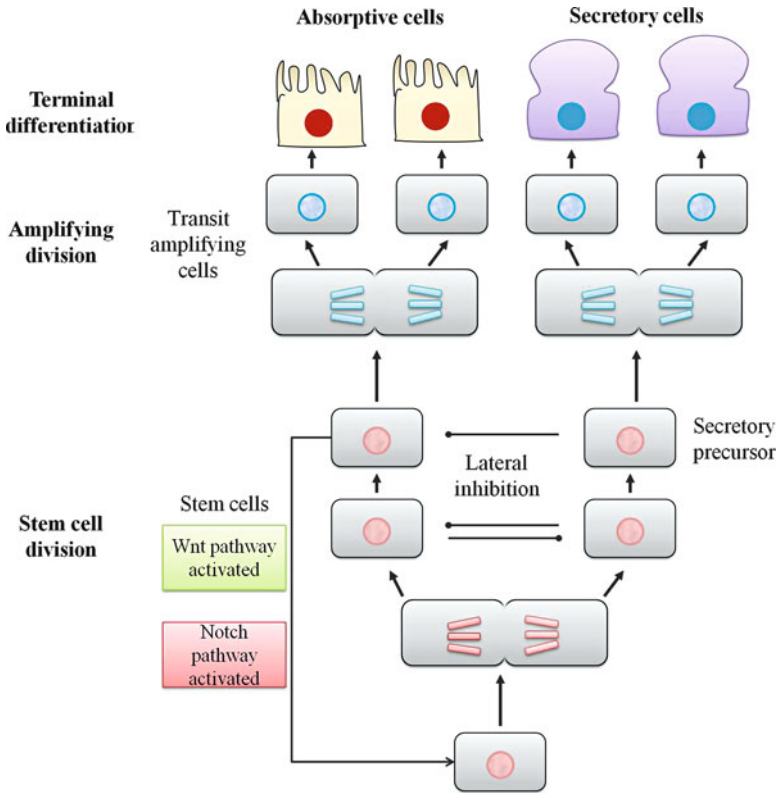


Fig. 14.5 Wnt and Notch signaling cooperatively maintain homeostasis in GI epithelium. Wnt and Notch signalling cooperate to maintain GI stem cells. Controlled activation of Wnt and Notch signaling parways in a cooperative manner help to maintain normal GI stem cell homeostasis, supplying progenitor/precursor cells which through further amplification can be terminally differentiated to absorptive and secretory cells

Desert hedgehog [DHH]), undergo auto processing and lipid modification to generate mature peptides (Katoh and Katoh 2005a). SHH-dependent parietal cell proliferation is implicated in gastric mucosal repair during chronic *Helicobacter pylori* infection (Katoh and Katoh 2005b). Hedgehog signaling also promotes epithelial proliferation in the esophagus, stomach and pancreas, and is thus frequently activated in esophageal, gastric and pancreatic cancer due to transcriptional up regulation of Hedgehog ligands and epigenetic silencing of the *HHIP1* gene. Hedgehog signaling inhibits proliferation in the intestine and is rarely activated in colorectal cancer due to negative regulation by the canonical Wnt signaling pathway.

Constant proliferation of GI stem cells increase the chance of mutation driven expansion of the altered stem cells and tumor progression. This provides a rationale for targeting tumor stem cells as being the most effective way to treat cancers such as colon cancer. These ideas have drawn attention to pathways that control

stem-cell proliferation as therapeutic targets. Among these, the TGF-β, Wnt, and Hedgehog pathways are of particular relevance to cancer. Recent data also suggest crosstalk between the Wnt and TGF-β pathways. While the Wnt signaling imposes a proliferative phenotype in crypt stem cells, the linear migration, differentiation and compartmentalization of cells along the crypt–villus axis is controlled by TGF-β and Wnt gradients. On the other hand, Hedgehog and Wnt counteract in the intestinal epithelium where Hedgehog inhibits Wnt signaling in intestinal stem or progenitor cells partly due to SFRP1 induction in mesenchymal cells. However, the mechanism of Wnt dependent Hedgehog signaling inhibition remains unclear (Katoh and Katoh 2006).

14.6 Gastro Intestinal Cancer Stem Cells

Increasing evidence in the last 10 years, shows that Cancer Stem Cells (CSCs) may play a critical role in tumor development and progression. CSCs are defined as a small subset of cancer cells that constitute a pool of self-sustaining cells with the exclusive ability to maintain the tumor. Currently, there are two hypothetical explanations for the existence of CSCs suggesting that they may arise either from normal stem cells by mutation of genes that render the stem cells cancerous or from differentiated tumor cells that experience further genetic alterations and, therefore, become dedifferentiated and acquire CSC-like features (Ariff and Eng 2005).

In the last decade, evidence has become available to indicate that CSCs are not only involved in the maintenance of hematopoietic tumors, but also in various solid cancers, including those of the breast, brain, prostate, colon and liver (Burra et al. 2011). CSCs have been identified based on the expression of various surface markers in different organs including CD 44 in stomach (Takaishi et al. 2008, 2009), and CD44, EpCam, CD166 (ALCAM), Prominin-1 (CD133) in Colon (Dalerba et al. 2007; Ricci-Vitiani et al. 2007; O'Brien et al. 2007; Shmelkov et al. 2008; Vermeulen et al. 2008).where as CD44, CD90, CD45, Prominin-1 in liver (Yang et al. 2008; Mishra et al. 2009) and CD44, CD24, antiepithelial-specific antigen (ESA), Prominin-1 in pancreas (Li et al. 2007; Hermann et al. 2007). In addition, it has been reported that intrahepatic stem cells, such as oval cells, can give rise to human hepatocellular carcinoma (HCC) and cholangiocarcinoma (Alison 2005). Recent literature also demonstrated presence of circulating cancer stem cells as diagnostic or prognostic markers (Nuh et al. 2010). It is now thought that targeting cancer stem cells may permit effective therapy in the treatment of cancer. This view was supported by recent studies reporting that a combination of blocking both sonic hedgehog and mTOR (mammalian target of rapamycin) signaling and standard chemotherapy seemed to eliminate pancreatic cancer stem cells (Mueller et al. 2009). However, much remains to be learned about these cancer stem cells and further research is needed to define the best markers and model systems used for studies of cancer stem cell populations in gastrointestinal tract.

14.7 Potential Applications of Gastro Intestinal Stem Cells

Stem cells represent a potential source of gastro intestinal epithelial cells and hepatocytes for a wide range of applications such as therapeutic applications, understanding developmental biology, disease modeling and drug toxicity testing. The intestinal epithelium is continually replaced by local stem cells (MacDonald et al. 1964; Toner et al. 1971; Cao et al. 2011). For the last 5 years, progress has been made in the identification and understanding GI stem cells, which have paved the way for exploring novel therapeutic approaches for gastrointestinal disease. However, further information regarding markers to assist in the identification and purification of stem cell populations and techniques to manipulate the cells both in vivo and in vitro is required. Because intestinal transplantation for patients still represents a significant challenge, it is hoped that in near future tissue-engineered intestine will provide a feasible option for patients with short bowel syndrome (Bitar and Raghavan 2012).

Therapeutic application of stem cells in GI disease: Stem cells are gaining importance for the treatment of chronic liver diseases and inflammatory or immune-mediated bowel diseases. Despite the fact that their self-renewing property is a high risk factor of carcinogenesis (Shackleton et al. 2009), hematopoietic stem cells (HSCs) transplantation was demonstrated to result in the remission or reduction of the inflammatory bowel disease (Kountouras et al. 2011), refractory Crohn's disease (Cassinotti et al. 2008) and celiac disease (Al-toma et al. 2007) with negligible adverse effects. Another group suggested that hematopoietic stem cells transplantation (HSCT) can be considered a reasonable option for patients who have failed standard CD therapy (Atkins et al. 2012). Recently the mesenchymal stem cell therapy is gaining importance in Crohn's fistulas in humans and beneficial effects of mesenchymal stem cells (MSCs) was also demonstrated in animal models of gastric and colonic ulcers, and intestinal bowel disease (González et al. 2009). Due to their lack of immunogenicity and ability to differentiate in to tissue specific cell types, MSCs are being considered to have comparatively more potential for use in regenerative therapy in many GI diseases (Satija et al. 2009). Similarly, autologous transplantation of bone marrow derived stem cells (BM-SC) improved liver function in patients with cirrhosis and hepatocellular carcinoma (HCC) after resection of liver prior to surgery (Ismail et al. 2010). Treatment with G-CSF was found to induce the proliferation of endogenous liver stem cells (Spahr et al. 2008) which can expedite the healing process. So far, most of the therapeutic trials in liver disease involving stem cells have limitations of small patient numbers and lack of controls. In liver diseases, the proof of efficacy of stem cell based therapies requires well-designed and adequately powered clinical trials. Although induced pluripotent stem cells have the ability to differentiate into hepatocytes *in vitro* (Song et al. 2009) with demonstrated therapeutic efficacy in animal models, their therapeutic application remains challenging. Another important observation with greater impact of GI stem cell therapy was in the area of gastrointestinal motility disorders, particularly those associated with the aganglionic gut (Metzger et al. 2009).

Developmental Biology: Stem cells could be used to study early events in development human gut epithelium. This may help researchers find out why some cells become cancerous and how some genetic diseases develop. This knowledge may indicate some clues about how these diseases may be prevented. Recently, mouse embryonic stem cells have been differentiated into gut-like structures which are useful to study the developmental mechanisms and diseases of the gastrointestinal tract (Torihashi et al. 2006).

Drug Toxicity Screening: Stem cells grown in the laboratory may be useful for testing drugs and chemicals before clinical trials. Stem cells could be directed to differentiate into specific cell types that are important for screening a specific drug. In comparison to animal models, these cells may be more likely to mimic the response of human tissue to the drug being tested. This makes drug testing a more safer, cheaper and ethically more acceptable approach addressing the apprehension of those who oppose the use of animals in pharmaceutical testing. It has been reported that human pluripotent stem cells such as embryonic and induced pluripotent stem cells can be differentiated into intestinal tissue in vitro. These pluripotent derived human intestinal tissues could help in drug toxicity studies in gastro intestinal diseases

14.8 Future Perspectives

Over the recent past, remarkable progress has been made in understanding stem cell biology specially focusing on tissue-specific adult stem cells. Although stem cells, have the regenerative potential for replacing cells lost in acute and chronic liver diseases and inflammatory or immune-mediated bowel diseases, the safety and efficacy of stem cells in therapeutic applications needs careful evaluation. Long term follow up studies are required to rule out the risk of malignant transformation and/or pro-fibrogenic effects of stem cell based therapies for gastro intestinal disorders. Future research may focus on

(i) identifying factors that control proliferation and differentiation of epithelial stem cells by establishing an in vitro model system
(ii) replacement of degenerative human tissues by newly synthesized genetically identical equivalents eliminating the need for organ donation and implantation of prosthesis
(iii) Major potential therapeutic benefit could arise from understanding mechanisms underlying ordered cell proliferation and differentiation.
(iv) Stem cell regeneration with respect to the complex environment of gut microbial flora.

Perhaps, insight into the protective mechanisms at play in the small bowel (which render it far less susceptible to tumorigenesis than the large bowel) will bring forth new therapeutics for gut cancers. Adopting scientific methods based on randomized and controlled trials should produce the necessary results on the real therapeutic role of stem cells.

References

Alison MR (2005) Liver stem cells: implications for hepatocarcinogenesis. Stem Cell Rev 1: 253–260

Altmann GG (1983) Morphological observations on mucus-secreting nongoblet cells in deep crypts of the rat ascending colon. Am J Anat 167:95–117

Altmann GG (1990) Renewal of intestinal epithelium: new aspects as indicated by recent ultrastructural observations. J Electron Microsc Technol 16:2–14

Al-toma A, Visser OJ, van Roessel HM et al (2007) Autologous hematopoietic stem cell transplantation in refractory celiac disease with aberrant T cells. Blood 109:2243–2249

Ariff B, Eng HL (2005) Stem cell: from bench to bedside, 1st edn. World Scientific Publishing, Singapore, pp 1–10

Atkins HL, Muraro PA, van Laar JM et al (2012) Autologous hematopoietic stem cell transplantation for autoimmune disease-is it now ready for prime time? Biol Blood Marrow Transplant 18(1 Suppl):S177–S183

Batlle E (2008) A new identity for the elusive intestinal stem cell. Nat Genet 40:818–819

Bitar KN, Raghavan S (2012) Intestinal tissue engineering: current concepts and future vision of regenerative medicine in the gut. Neurogastroenterol Motil 24(1):7–19

Bjerknes M, Cheng H (2005) Gastrointestinal stem cells. II. Intestinal stem cells. Am J Physiol Gastrointest Liver Physiol 289:G381–G387

Burra P, Bizzaro D, Ciccocioppo R (2011) Therapeutic application of stem cells in gastroenterology: an up-date. World J Gastroenterol 17(34):3870–3880

Cao L, Gibson JD, Miyamoto S (2011) Intestinal lineage commitment of embryonic stem cells. Differentiation 81(1):1–10

Cassinotti A, Annaloro C, Ardizzone S et al (2008) Autologous haematopoietic stem cell transplantation without CD34+ cell selection in refractory Crohn's disease. Gut 57:211–217

Chang WWL, Leblond CP (1971) Renewal of the epithelium in the descending colon of the mouse. I. Presence of three cell populations: vacuolated-columnar, mucous and argentaffin. Am J Anat 131:73–100

Chang WWL, Nadler NJ (1975) Renewal of the epithelium in the descending colon of the mouse. IV. Cell population kinetics of vacuolated-columnar cells. Am J Anat 144:39–58

Cheng H, Leblond CP (1974a) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cells. Am J Anat 141:461–480

Cheng H, Leblond CP (1974b) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. III. Entero-endocrine cells. Am J Anat 141:503–520

Dalerba P, Dylla SJ, Park I (2007) Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci USA 104:10158–10163

El-Alfy M, Leblond CP (1987a) Long duration of mitosis and consequences for the cell cycle concept, as seen in the isthmal cells of the mouse pyloric antrum. I. Identification of early and late steps of mitosis. Cell Tissue Kinet 20:205–213

El-Alfy M, Leblond CP (1987b) Long duration of mitosis and consequences for the cell cycle concept, as seen in the isthmal cells of the mouse pyloric antrum. II. Duration of mitotic phases and cycle stages, and their relation to one another. Cell Tissue Kinet 20:215–226

El-Alfy M, Leblond CP (1989) An electron microscopic study of mitosis in mouse duodenal crypt cells confirms that prophasic condensation of chromatin begins during the DNA- synthesizing (S) stage of the cycle. Am J Anat 186:69–84

Gebert A, Posselt W (1997) Glycoconjugate expression defines the origin and differentiation pathway of intestinal M-cells. J Histochem Cytochem 45:1341–1350

González MA, Gonzalez-Rey E, Rico L et al (2009) Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology 136:978–989

He XC, Zhang J, Tong WG et al (2004) BMP signaling inhibits intestinal stem cell self renewal through suppression of Wnt-beta-catenin signaling. Nat Genet 36:1117–1121

Hermann PC, Huber SL, Herrler T et al (2007) Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 1:313–323
Ismail A, Fouad O, Abdelnasser A et al (2010) Stem cell therapy improves the outcome of liver resection in cirrhotics. J Gastrointest Cancer 41:17–23
Karam SM, Leblond CP (1993a) Dynamics of epithelial cells in the corpus of the mouse stomach. I. Identification of proliferative cell types and pinpointing of the stem cell. Anat Rec 236:259–279
Karam SM, Leblond CP (1993b) Dynamics of epithelial cells in the corpus of the mouse stomach. II. Outward migration of pit cells. Anat Rec 236:280–296
Karam SM, Leblond CP (1993c) Dynamics of epithelial cells in the corpus of the mouse stomach. III. Inward migration of neck cells followed by progressive transformation in to zymogenic cells. Anat Rec 236:297–313
Karam SM, Leblond CP (1993d) Dynamics of epithelial cells in the corpus of the mouse stomach. V. Behaviour of entero-endocrine and caveolated cells. General conclusions on cell dynamics in the oxyntic epithelium. Anat Rec 236:333–340
Karam SM, Li O, Gordon JI (1997) Gastric epithelial morpho-genesis in normal and transgenic mice. Am J Physiol 272:G1209–G1220
Katoh Y, Katoh M (2005a) Comparative genomics on Sonic hedgehog orthologs. Oncol Rep 14:1087–1090
Katoh Y, Katoh M (2005b) Hedgehog signaling in gastric cancer. Cancer Biol Ther 4:1050–1054
Katoh U, Katoh M (2006) Hedgehog signaling pathway and gastrointestinal stem cell signaling network. Int J Mol Med 18:1019–1023
Kosinki C, Li VS, Chan AS et al (2007) Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. Proc Natl Acad Sci USA 104:15418–15423
Kountouras J, Sakellari I, Tsarouchas G et al (2011) Autologous haematopoietic stem cell transplantation in a patient with refractory Crohn's disease. J Crohns Colitis 5(3):275–276
Leblond CP, Stevens CE, Bogoroch R (1948) Histological localization of newly-formed desoxyribonucleic acid. Science 108:531–533
Lee ER (1985) Dynamic histology of the antral epithelium in the mouse stomach: II. Ultrastructure and renewal of pit cells. Am J Anat 172:225–240
Lee ER, Leblond CP (1985a) Dynamic histology of the antral epithelium in the mouse stomach: I. Ultrastructure and renewal of isthmal cells. Am J Anat 172:205–224
Lee ER, Leblond CP (1985b) Dynamic histology of the antral epithelium in the mouse stomach: III. Ultrastructure and renewal of gland cells. Am J Anat 172:241–259
Li L, Clevers H (2010) Coexistence of quiescent and active adult stem cells in mammals. Science 327:542–545
Li YQ, Roberts SA, Paulus U et al (1994) The crypt cycle in mouse small intestinal epithelium. J Cell Sci 107:3271–3279
Li Q, Karam SM, Gordon J (1995) Simian Virus 40 T antigen amplification of pre-parietal cells in transgenic mice: effects on other gastric epithelial cell lineages and evidence for a p53-independent apoptotic mechanism that operates in a committed progenitor. J Biol Chem 270:15777–15788
Li C, Heidt DG, Dalerba P et al (2007) Identification of pancreatic cancer stem cells. Cancer Res 67:1030–1037
MacDonald WC, Trier JS, Everett NB (1964) Cell proliferation and migration in the stomach, duodenum, and rectum of man: radioautographic studies. Gastroenterology 46:405–417
Maunoury R, Robine S, Pringault E et al (1992) Developmental regulation of villin gene expression in the epithelial cell lineages of mouse digestive and urogenital tracks. Development 115:717–728
Merritt AJ, Allen TD, Potten CS et al (1977) Apoptosis in small intestinal epithelium from p53-null mice: evidence for a delayed, p53-independent G2/M-associated cell death after γ -irradiation. Oncogene 14:2759–2766
Metzger M, Caldwell C, Barlow AJ et al (2009) Enteric nervous system stem cells derived from human gut mucosa for the treatment of aganglionic gut disorders. Gastroenterology 136:2214–2225

Mills JC, Syder AJ, Hong CV et al (2001) A molecular profile of the mouse gastric parietal cell with and without exposure to Helicobacter pylori. Proc Natl Acad Sci USA 98(24):13687–13692
Mishra L, Shetty K, Tang Y et al (2005) The role of TGF-b and Wnt signaling in gastrointestinal stem cells and cancer. Oncogene 24:5775–5778
Mishra L, Banker T, Murray J et al (2009) Liver stem cells and hepatocellular carcinoma. Hepatology 49:318–329
Moore KA, Lemischka IR (2006) Stem cells and their niches. Science 311:1880–1885
Mueller MT, Hermann PC, Witthauer J et al (2009) Combined targeted treatment to eliminate tumorigenic cancer stem cells in human pancreatic cancer. Gastroenterology 137:1102–1113
Nuh N, Rahbari MA, Kristian T et al (2010) Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer. Gastroenterology 138: 1714–1726
O'Brien CA, Pollett A, Gallinger S et al (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445:106–110
Pinto D, Gregorieff A, Begthel H et al (2003) Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes Dev 17:1709–1713
Potten CS (1998) Stem cells in gastro intestinal epithelium: numbers, characteristics and death. Philos Trans R Soc Lond 353:821–830
Potten CS, Hendry JH (1995) Clonal regeneration studies. In: Radiation and gut. Elsevier, Amsterdam, pp 45–59
Potten CS, Loeffler M (1990) Stem cells: attributes, cycles, spirals, pitfalls and uncertainties: lessons for and from the crypt. Development 110:1001–1020
Potten CS, Booth C, Pritchard DM (1997) The intestinal epithelial stem cell: the mucosal governor. Int J Exp Pathol 78:219–243
Potten CS, Owen G, Booth D (2002) Intestinal stem cells protect their genome by selective segregation of template DNA strands. J Cell Sci 115:2381–2388
Quante M, Wang TC (2009) Stem cells in gastroenterology and hepatology. Nat Rev Gastroenterol Hepatol 6(12):724–737
Ricci-Vitiani L, Lombardi DG, Pilozzi E et al (2007) Identification and expansion of human colon-cancer-initiating cells. Nature 445:111–115
Sancho E, Batlle E, Clevers H (2003) Live and let die in the intestinal epithelium. Curr Opin Cell Biol 15:763–770
Satija NK, Singh VK, Verma YK et al (2009) Mesenchymal stem cell-based therapy: a new paradigm in regenerative medicine. J Cell Mol Med 13:4385–4402
Sato M, Ahnen D (1992) Regional variability of colonocyte growth and differentiation in the rat. Anat Rec 233:409–414
Sato A, Spicer SS (1980) Ultrastructural cytochemistry of complex carbohydrates of gastric epithelium in the guinea pig. Am J Anat 159:307–329
Scoville DH, Sato T, He XC et al (2008) Current view: intestinal stem cells and signaling. Gastroenterology 134:849–864
Shackleton M, Quintana E, Fearon ER et al (2009) Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell 138:822–829
Shmelkov SV, Butler JM, Hooper AT et al (2008) CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumors. J Clin Invest 118:2111–2120
Song Z, Cai J, Liu Y et al (2009) Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. Cell Res 19:1233–1242
Spahr L, Lambert JF, Rubbia-Brandt L et al (2008) Granulocyte-colony stimulating factor induces proliferation of hepatic progenitors in alcoholic steatohepatitis: a randomized trial. Hepatology 48:221–229
Spradling A, Drummond BD, Kai T (2001) Stem cells find their niche. Nature 41:98–104
Takaishi S, Okumura T, Wang TC (2008) Gastric cancer stem cells. J Clin Oncol 26:2876–2882
Takaishi S, Okumura T, Tu S et al (2009) Identification of gastric cancer stem cells using the cell surface marker CD44. Stem Cells 27:1006–1020

Toner PG, Carr KE, Wyburn GM (1971) The digestive system: an ultrastructural atlas and review, vol 76. Butterworth, London, pp 149–150
Torihashi S, Kuwahara M, Ogaeri T et al (2006) Gut-like structures from mouse embryonic stem cells as an in vitro model for gut organogenesis preserving developmental potential after transplantation. Stem Cells 24(12):2618–2626
Troughton WD, Trier JS (1969) Paneth and goblet cell renewal in mouse duodenal crypts. J Cell Biol 41:251–268
Tsubouchi S, Leblond CP (1979) Migration and turnover of entero- endocrine and caveolated cells in the epithelium of the descending colon, as shown by radioautography after continuous infusion of 3H- thymidine into mice. Am J Anat 156:431–452
Vermeulen L, Todaro M, De souse Mello F et al (2008) Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. Proc Natl Acad Sci 105:13427–13432
Yang ZF, Ngai P, Ho DW et al (2008) Identification of local and circulating cancer stem cells in human liver cancer. Hepatology 47:919–928
Yen TH, Wright NA (2006) The gastrointestinal tract stem cell niche. Stem Cell Rev 2(3):203–212

Chapter 15
Cancer Stem Cells

Murali M.S. Balla, Anjali P. Kusumbe, Geeta K. Vemuganti, and Sharmila A. Bapat

Abstract Our knowledge of carcinogenesis has tremendously improved through decades of research. However, till date the therapeutic refractoriness and tumor dormancy that leads to cancer recurrence after therapy presents formidable obstacles through severely limiting the successful treatment outcomes for majority of cancers. Significant advances made recently in the cancer stem cell (CSC) biology field have provided new insights into cancer biology that are radically changing both our understanding of carcinogenesis and cancer treatment. The cancer stem cell hypothesis provides an attractive cellular mechanism to account for the therapeutic refractoriness and dormant behavior exhibited by many of these tumors. Direct evidence for the CSC hypothesis has recently emerged through their identification and isolation in diverse tumor types. These tumor types appeared to be hierarchically organized and sustained by a distinct fraction of self-renewing and tumor-initiating CSCs. Such illustration of the CSC paradigm in diverse tumor types necessitates reassessment and improvisation of the current therapeutic strategies

M.M.S. Balla
Champalimaud Translational Centre for Eye Research, Hyderabad Eye Research Foundation, L.V. Prasad Eye Institute, L.V. Prasad Marg, Banjara Hills, Hyderabad 500 034, Andhra Pradesh, India

A.P. Kusumbe
National Centre for Cell Science, NCCS Complex, Ganeshkhind, Pune 411 007, Maharashtra, India

G.K. Vemuganti, M.D., DNB, FAMS, FICP
School of Medical Sciences, University of Hyderabad, Prof C R Rao Road, Gachibowli, Hyderabad 500 046, Andhra Pradesh, India

S.A. Bapat, Ph.D., FNASc (✉)
National Centre for Cell Science, NCCS Complex, Pune University Campus, Ganeshkhind, Pune 411 007, Maharashtra, India
e-mail: sabapat@nccs.res.in

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_15,

originally developed against the homogenous tumor mass; now to specifically target the CSC population. Preliminary findings in the field indicate that such specific targeting of CSCs may be possible.

Abbreviations

CSCs	Cancer stem cells
NSCs	Normal stem cells, HSCs, Hematopoetic stem cells
RB	Retinoblastoma
LRCs	Label retaining cells
EMT	Epithelial to mesenchymal transition
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
SP	Side population
MDR	Multidrug resistance

15.1 Introduction

Cancer pathogenesis has been recognized since a long time to be multi-step process at the phenotypic and genetic levels. Several factors including non-lethal triggers (chemical carcinogens, radiation and viral infections) or inherited/*de novo* genetic alterations in the form of mutations and other genetic rearrangements underlie this multi-step progression. Such changes drive carcinogenesis through activating growth promoting oncogenes, altering apoptosis regulating genes and/or inactivating tumor suppressor genes that culminate in a loss of cell cycle regulation and unregulated clonal expansion of cells. Further additional and diverse mutation hits in such cell clones results in a malignant neoplasm characterized by functional heterogeneity (Cotran 1994). Further the cellular content of tumors has also been speculated to be heterogeneous with only a small fraction of whole tumor driving its generation and disease progression. Supporting this hypothesis, the cancer stem cell (CSC) model posits that many human malignancies consist of two functionally distinct cell types: (i) CSCs that are self-renewing cells with the capacity to initiate, sustain, and lead to disease progression; and (ii) non-self-renewing progeny cells, derived from CSCs through differentiation that make up the bulk of the tumor and account for disease symptomatology (Al-Hajj et al. 2003; Bonnet and Dick 1997; Cobaleda et al. 2000; Lapidot et al. 1994; Bapat 2009).

Although the concept of CSCs was postulated decades ago, the first evidence for their existence was provided through clonal assays in acute myeloid leukemia (AML), wherein the similarity was extended to the hierarchical organization of leukemic cells in much the same manner as normal hematopoietic tissues (Lapidot et al. 1994). CSCs are thought to sit at the apex of cellular hierarchy within a tumor and may be responsible for disease initiation and for relapse. By analogy with normal

hematopoietic stem cells (HSCs), they are predicted to be relatively quiescent and resistant to conventional chemotherapy.

The above study paved the path to further isolation of CSC fractions from solid tumors. Today compelling data support the CSC model in various human cancers including breast, brain, colon, pancreas, prostate and ovary (Bapat 2009; Li et al. 2007; O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Singh et al. 2004; Yilmaz et al. 2006). The main criteria that formed the basis of characterization of CSCs included immunophenotyping, clonal assays, tumor regeneration ability in animals and gene expression patterns. In each case, CSCs were identified as a rare cellular fraction within tumors, which on transplantation into immunocompromised mice generated new tumors with similar pathological features as that of the original tumor. Additional studies have also demonstrated the presence of minor population of 'stem-like' cells in well-established cancer cell lines (Hirschmann et al. 2004; Setoguchi et al. 2004).

Further characterization has also revealed CSCs to be therapeutic refractory and crucial determinants of tumor recurrences. Thus, for achieving long-term disease free survival efficient cancer therapy should be able to eradicate CSC fraction. However targeting this CSC fraction requires their detailed characterization in terms of their genotype, phenotype, epigenetic and functionality. The current chapter provides an overview of the emerging evidences and concepts in CSC biology.

15.2 Concept of Carcinogenesis Based on Cancer Stem Cells

Tumor formation is a complex multi-factorial process influenced by myriad cues including host responses elicited on transformed cells or their products, doubling time of tumor cells, tumor – niche interactions, angiogenesis, etc. It is evident that by the time a tumor is detected, it has already undergone several events most significantly those influencing the cell cycle. Thus, it is estimated at initial diagnosis, a tumor weighing around 1 g portion has probably undergone approximately 30 population doublings that results in generation of 10^9 cells (Cotran 1994). The cell cycle of tumors, like that of normal cells, has same five phases; G0, G1, S, G2, and M. With expanding cell populations during the disease progression a higher percentage of tumor cells either leave the proliferative/replicative pool by reversion to G0 phase or divide at very slow rate, while the remaining differentiate and latter perish (Cotran 1994).

In the context of regenerative tumor hierarchies, the initial trigger/causative transformation event could occur in a proliferation competent adult stem cell. Subsequent mutational hits in the same cells are necessary to make it competent enough to overcome the natural tissue elimination process. Additionally, a differentiated cell through gain of self-renewal capacity as a consequence of mutation hits, can also initiate neoplastic growth. In a majority of cases, tumors regenerated from such mutant cells through repetitive divisions, appear to be predominantly monoclonal (Cotran 1994). During disease progression, additional mutations occur in the tumor cells, contributing

to poor prognostic features, as evident in the mutation screening studies of *RB1* in Indian patients at L.V. Prasad Research Institute (Kiran et al. 2003). Distinct proliferating and differentiated cellular compartments can be identified in majority of tumor types (Cotran 1994). The proliferating fraction (cells in S, G2 and M phase) within tumors has a profound effect on their susceptibility to cancer chemotherapy as most anticancer drugs act only on proliferating pool. In this context, a tumor with a consistent cellular fraction that exit the cell cycle and are maintained in the G0 phase or are slowly growing will be relatively refractory to such treatments (Cotran 1994). It is been demonstrated that CSCs occupy this quiescent cellular fraction that is maintained in the G0 phase (Kusumbe and Bapat 2009).

Further series of investigations in the field identified phenotypic and functional similarities between normal stem cells (NSCs) and CSCs. Since NSCs and CSCs share the ability to self-renew, the machinery for self-renewing cell division is also likely to be similar in these two cell types. Accordingly, evidence indicating that several pathways that are classically associated with NSCs including Sox2, Nanog, Oct4, Wnt, Notch, Sonic Hedgehog, BMI-1 and EZH2 (Duncan et al. 2005; Hopfer et al. 2005; Katano 2005; Kolligs et al. 2002; Rask et al. 2003; Reya et al. 2001; Sanchez et al. 2005; Wilson and Radtke 2006) also regulate self-renewal in CSCs. Akin to NSCs, CSCs possess elongated telomeres, increased telomerase activity, express ABC transporters which confers resistance to chemotherapeutic drugs, proliferation capability in absence growth factors, trigger neo-angiogenesis by secreting angiogenic factors and express receptors and adhesion molecules (such as CXCR4, LIF-R, c-met, c-kit) associated with homing and metastasis (Duncan et al. 2005; Katano 2005; Kolligs et al. 2002; Peeters et al. 2006; Zagzag et al. 2005).

Cellular heterogeneity within tumor tissues has been one of the initial observations in the study of cancer, and the cellular mechanisms underlying such tumor heterogeneity has remained a subject of intense research in the field. In such a heterogeneous tumor, mass identification of the cell type capable of sustaining the growth of neoplasm clone presents a major problem. Although there are enough evidences that indicate virtually all cancers are clonal in origin and represent the progeny of a single cell (Fialkow 1976; Fearon et al. 1987) what has remained a fundamental problem in cancer is which cells within the tumor have the ability to seed another tumor? The concept that not every cell within the tumor clone possesses ability to seed another tumor stems from the following observations. In 1973, McCulloch and colleagues observed that only 1 in 100 to 1 in 10,000 murine myeloma cells had the ability to form colonies *in vitro*. Similarly, when several thousands of cells obtained from primary solid tumors were seeded in soft-agar, the colony forming efficiency was found to be between 0.1 and 0.5 % (Hamburger and Salmon 1977). Additionally, a series of transplantation experiments demonstrated that autologous injection of tumor cells subcutaneously requires at least 106 cells to initiate tumor formation (Southam and Brunschwig 1960). Furthermore, pioneering studies of spontaneous mouse leukaemias and lymphomas revealed that the frequency of tumour-propagating cells ranged from 1 % to the majority of cells (Hewitt 1958). Thus, these observations raise a fundamental question: Why not every cell within a tumor mass is capable of initiating a new tumor? At least two models have

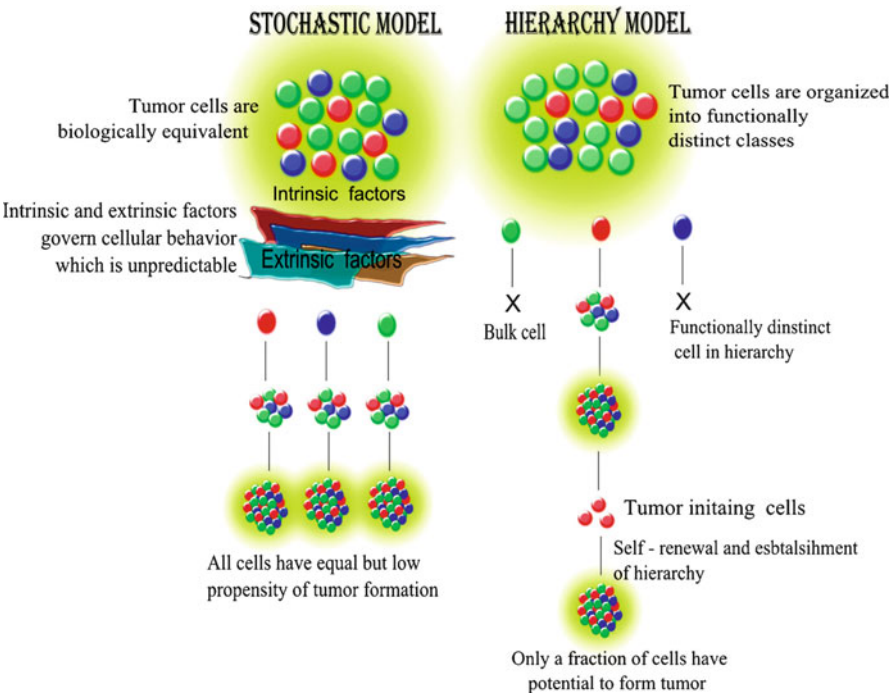


Fig. 15.1 Schematic illustrating the two models: stochastic model and hierarchy model for accounting tumor cellular heterogeneity

been proposed to account for the cellular heterogeneity and inherent differences in tumor-regenerating capacity: stochastic and hierarchy model (Fig. 15.1).

15.2.1 Stochastic Model

The stochastic model postulates that the heterogeneous cellular content of tumors is equivalent at a biological level but differential at behavioral level. Since behavior is influenced by extrinsic as well as intrinsic factors, it remains largely unpredictable. Consequently cells with clone/tumor initiating capability may not be enriched. Thereby, the stochastic model proposes that all the cells have equal but low propensity of tumor formation (Nowell 1976; Kruh 2003).

15.2.2 Hierarchy Model

The hierarchy model on the other hand proposes that tumors are heterogeneous. Further only rare, limited number of cells within a tumor actually have the clonogenic potential to inititate tumor regeneration. Thereby, while according to stochastic

model, the choice of cells initiating a new tumor is through chance, in the hierarchy model it is a pre-ordained event. As per the hierarchy model there exist a distinct functional heterogeneity among the cells that comprise a tumor – only few cells within the tumor are actually clonogenic and tumorigenic (now termed as CSCs), while the bulk majority is the non-tumorigenic fraction. Current emerging evidence strongly supports the hierarchy model (Lapidot et al. 1994; Bapat 2009).

Although the CSC hypothesis provides an attractive model to account for the cellular heterogeneity within tumors it does not identify the cellular origin of CSCs. Three possible origins of CSCs as listed below have been suggested (Box. 15.1); however till date their exact cellular origin remain unproved.

Box 15.1 Cellular Origin of CSCs

Mutations in stem cells leading to uncontrolled self-renewal

Adult stem cells initially seemed to be the most probable origin for CSCs due to following reasons:

(i) These are the only cells that persist for long periods upto several decades within a tissue thereby allowing the accumulation of series of mutations;
(ii) Since stem cells have indigenous machinery for maintaining the self-renewal state, it is simpler to continue with an ongoing program than turning it on in more mature cell types. Consequently fewer mutational hits would be required transform stem cells compared to their mature counterparts.

Mutations in early progenitors leading to acquisition of a dysregulated self-renewal state

Although stem cells seem to be the most probable candidates for CSC origin, it has been shown that CSC can also arise from early progenitors. The latter are derived from original stem cells that have undergone minimal number of divisions, and hence they have a high possibility of acquiring stem cell properties with minimal transformations. The early progenitor cell may be transformed either by acquiring mutations that leads to self renewal like stem cells or by inheritance of existing mutations in stem cells such that minimum (one or two) mutation event/s are required to mediate their transformation.

De-differentiation of late transit amplifying or differentiated somatic cells into immortal stem cells

Committed progenitors and differentiated cells would necessitate several mutational hits for the achievement of self-renewal and immortal state as compared to other cells in the hierarchy hence remained the least speculated origin for CSCs. However, recent evidences suggest that even committed progenitors can undergo de-differentiation and reacquire the property of self-renewal to give rise to stem like cells.

15.3 Isolation and Characterization of CSCs

Characterization of CSCs from tumor tissue can be viewed as the first step on a roadmap leading to identification of therapeutic targets against these cellular entities towards facilitating their eradication. Therefore all efforts should now be focused on their appropriate identification and isolation of CSCs from tumor tissue. However, as tumor tissues are heterogeneous containing mixture of self renewing CSCs, transiently amplifying progenitors and proliferative cells that can undergo differentiation resulting in various cell types (Jordan et al. 2006; Krivtsov et al. 2006) identification and isolation of CSCs is a challenging problem in cancer biology.

15.3.1 Surface Marker Expression Analysis

Stem cells from somatic tissue have been best characterized in the hematopoietic system, based upon the cluster of differentiation (CD) cell surface marker expression. Further easy accessibility of blood forming cells and availability of wide range of well-characterized *in vitro* and *in vivo* functional assays provides additional technical advantages. Thus it is not surprising that first experimental characterization of CSCs came from acute myeloid leukemia. Specifically this study demonstrated that a rare subset of cells expressing a similar surface marker expression ($CD34^+/CD38^-$) as of primitive bone marrow cells was the only subset endowed with clonogenic potential within total AML population (Bonnet and Dick 1997; Cobaleda et al. 2000; Lapidot et al. 1994). This approach was further extended to solid tumors such as breast, brain, prostrate lung, pancreas, liver and colorectal tumors (Li et al. 2007; Ricci-Vitiani et al. 2007; Singh et al. 2004; Al-Hajj et al. 2003) where cell surface markers of normal stem cells formed the basis of CSCs identification and characterization (Table 15.1).

Such phenotypic identification of CSCs from diverse tumor types through exploiting NSC markers suggests that current strategy for CSC identification from its respective cancer tissue mainly relies on surface marker expression analysis. However, applicability of this strategy in tumors from organs such as ovary where NSCs remain elusive and their markers as yet unidentified demands an alternative strategy to identify appropriate CSC markers. Thus several classical generic stem cell markers including CD133, c-kit, CD44, CD24, etc. have been proposed as markers for ovarian CSCs.

The validity of such an approach however is not certain in all the cases. Several CSC markers being applied currently for isolation of CSCs are not known to be expressed in the corresponding normal tissue, but may be present in other tissues. In a recent study, we analyzed expression of several such surface markers including CD133 which currently represents the most widely reported CSC marker. However, in our study, characterization of CD133 expressing cells from ovarian tumors revealed their identity as non-tumorigenic endothelial precursors (Kusumbe et al. 2009). Moreover, existence of phenotypic heterogeneity within CSC population has

Table 15.1 Surface markers identified in various tumors

Tumors/cell lines	Markers/stem cell like properties characterized	Ref.
Acute myeloid leukaemia	$CD34^+/CD38^-$, $CD90^-$, $ckit^-$; Tumorogenic *in vivo*	Lapidot et al. (1994)
Multiple myeloma	$CD138^-$; Tumorogenic *in vivo*	Matsui et al. (2004)
Brain tumors	$CD133^+$/$Nestin^+$; Neurosphere forming capability, Tumorogenic *in vivo*	Singh et al. (2004)
Prostrate	$CD133^+$, $CD44^+$, $\alpha 2\beta 1$ integrin ^{high}, spheres formed, SMO^+; Tumorogenic *in vivo*	Collins et al. (2005)
Breast	$CD44^+/CD24^-$, Oct4 ; Mammospheres formation observed, Tumorogenic *in vivo*	Al-Hajj et al. (2003)
Hepatocellular	$CD133^+$	Suetsugu et al. (2006)
Lung	$Sca1^+$, $CD45^-$, $CD31^-$, $CD34^+$	Kim et al. (2005)
Pancreas	$CD44^+/CD24^+/ESA^+$, $CD133^+$/ABCG2; Tumorigenic *in vivo*	Li et al. (2007)
Colorectal	$CD133^+$; Colon spheres, Tumorogenic *in vivo*	O'Brien et al. (2007)
Melanoma	$CD20^+$; Spheroid formation is observed	Grichnik et al. (2006)
Ovarian cancer	$CD44^+$, Oct3/4, Nanog, EGFR, Vimentin/Snail; Tumorogenic in vivo	Bapat et al. (2005)
Head and neck cancer	$CD44^+$; Tumorogenic *in vivo*	Prince et al. (2007)
Retinoblastoma	ABCG2, MCM2, $CD44^+$, $CD133^-$,$CD90^-$, Oct4, Nanog	Balla et al. (2009)

also been documented (Visvader and Lindeman 2008). Thus, although application of surface markers provides an important and convenient tool for CSC identification, these drawbacks demand switching to functional definitions in CSC identification schemes.

15.3.2 Clonogenic Assays

These assays were first carried out by Puck and Marcus (1956) for NSCs; the first attempt of agar assay on primary tumor cells were done with mouse myeloma (Puck et al. 1956; Ogawa et al. 1973) following which human myeloma and other tumor cells were also culture (Park et al. 1980; Courtenay and Mills 1978; Courtenay et al. 1978; Hamburger and Salmon 1977). These clonal assays paved the path towards establishment of a stem cell model of human tumor growth. The read-out is number of clone forming cells within a population of tumor cells thus providing the evidence of CSCs in the heterogeneous population of tumors. A long-term culture assay (where self renewal and clonogenic capacity forms the basis of assay) denotes the classical approach for HSC identification (Moore 1991). Eventually, clonogenic assays were used for clinical drug testing and pre clinical drug screening (Park et al. 1980; Courtenay and Mills 1978; Courtenay et al. 1978).

Adaptation of a similar approach should also enable identification and isolation CSCs from tumor tissue, as this is the only fraction endowed with self-renewal and indefinite proliferation potential. Such LTSC based existence of CSCs was first demonstrated in ovarian cancer (Bapat et al. 2005) in which, from a single multilayered layered spheroid derived from ascites of a Grade IV ovarian serous adenocarcinoma patient several single cell clones were isolated and subjected to long-term culture assay. Subsequently 19 cell clones formed proliferative colonies in this assay thereby claiming their CSC identity. Detailed marker analysis and functional assays confirmed this identity in two cell clones; later other three cell clones were demarcated as pre-tumorigenic CSCs (Wani et al. 2006) while remaining 14 were characterized to be non-tumorigenic endothelial stem cells (Kusumbe et al. 2009).

15.3.3 Side Population Analysis

An elegant strategy to isolate stem cells has been designed based on their ability to efflux dyes such as Hoechst 33342 and Rhodamine 123 from their cytoplasm (Decraene et al. 2005; Larderet et al. 2006; Challen and Little 2006). The isolation and characterization of cells by this method was initially described by Goodell et al. using mouse bone marrow (Goodell et al. 1996). When entire bone marrow populations were stained using Hoechst 33342 and observed simultaneously for fluorescence at two emission wavelengths (blue 450 nm and red 675 nm), cells with maximum efflux capability segregated as a side-population (SP) next to Hoechst bright population (Fig. 15.2). This SP fraction expressed stem cell markers ($Sca1^{+}$ $Lin^{-/low}$) and was capable of reconstituting the bone marrow of irradiated mice upon transplantation (Challen and Little 2006). Further the degree of efflux correlated well with the stemness state. Cells exhibiting the highest efflux are more primitive than cells that exhibit dye uptake and more differentiated (Goodell et al. 1996).

Hoechst 33342 is known to bind to the A-T-rich regions of the minor DNA groove, and the intensity of its fluorescence is a measure of DNA content, chromatin structure and cell cycle (Goodell et al. 1996). This effluxing mechanism demonstrated by SP has been proposed to result from expression of multidrug resistance (MDR) proteins, such as ABC transporters, that can actively pump several drugs out of a cell. This hypothesis was elucidated by using ATP-binding cassette inhibitor verapamil which showed that Hoechst 33342 exclusion by SP cells decreases on blockage of ATP-binding cassette transmembrane protein. However, MDR1 is found to be expressed by 65 % of bone marrow cells while the SP fraction represents only 0.1 % of total bone marrow population (Goodell et al. 1996). Thus the MDR expression alone cannot identify and distinguish SP cells. Further studies demonstrated breast cancer resistance protein to be a more specific marker for SP cells from breast tumors; in addition also the integrin β_3 expression correlated well with SP phenotype (Umemoto et al. 2006). The dye efflux strategy was first used to isolate the HSCs and latter was extended identification NSCs from various organs. SP has also been identified in cancer cell lines and diverse tumor types including neuroblastoma,

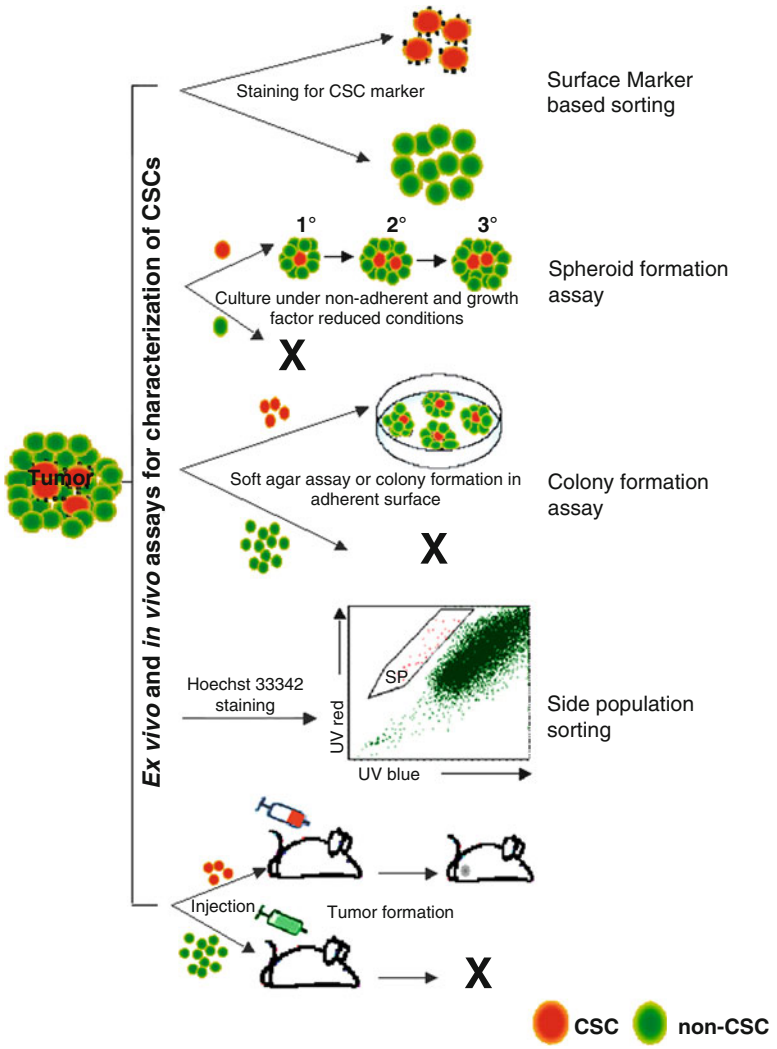


Fig. 15.2 Schematic representing the current ex vivo and in vivo strategies adapted for characterization of CSCs from blood cancers and diverse solid tumors. Cell surface marker expression based FACS sorting provides the most convenient tool for CSC isolation. However recent studies illustrating existence of phonotypical heterogeneity demands further validation of this approach. The non-adherent sphere assay predicts that CSC can be serially passaged for many cycles and that it generates a tumor sphere resembling the primary sphere in each case. Colony formation assays are also the readout of CSC activity. However CSCs and progenitors cannot be distinguished in these clonal assays. Side population assessment of the is based on the stemness specific functional ability of CSCs to actively efflux dyes such as Hoechst 33342 out of their cytoplasm due to the expression of MDR proteins, such as ABC transporters. The gold-standard functional assay for evaluating the presence of CSCs is transplantation of sorted subpopulations into immunocompromised mice models

melanoma, retinoblastoma, ovarian, hepatocellular carcinoma, and glioma (Chiba et al. 2006; Grichnik et al. 2006; Kondo et al. 2004; Szotek et al. 2006).

15.3.4 Tumor Forming Ability in Animals

The most convincing demonstration of CSC identity comes from serial transplantation of cellular populations into animal models, necessitating development of orthotopic transplantation assays. CSC-containing population is recognized from their capability to re-establish the phenotypic heterogeneity evident in primary tumours, and exhibit self-renewing capability on serial passaging. Even in the first studies with leukemia in 1997, the final validation of CSCs came from transplantation of a minority of undifferentiated cells isolated based on surface expression from AML patients into NOD/SCID (non-obese diabetic/severe combined immunodeficient) mice. This transplantation proved these cells to be the only subset within the total population capable of reconstituting tumors. Moreover, the resulting tumors included a range of more differentiated cell types as in the original human disease (Bonnet and Dick 1997). The same model CSCs were further used for characterization of several solid cancers. The first report for identification of CSCs in solid tumors came in breast cancer in which human breast cancer cells were transplanted in Non Obese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice. It was found that as few as 100 $CD44^{high}$ $CD24^{-/low}$ cells were able to form tumors in mice, whereas 10,000 of cells with alternate phenotypes failed to form tumors (Al-Hajj 2003). The tumorigenic subpopulation could be serially passaged; each time cells within this phenotype generated new tumors with additional $CD44^{high}/CD24^{-/low}$ tumorigenic cells as well as the phenotypically diverse mixed populations of nontumorigenic cells present in the original tumor. However recently there have been significant technical issues with this assay system mainly due incomplete immunosuppression. Additionally, species-specific differences in cytokines or growth factors present a confounding issue. Even in syngeneic models, implantation of tumor cells into a normal niche does not precisely recapitulate the tumor microenvironment itself (Visvader and Lindeman 2008).

15.4 Cancer Stem Cells in Retinoblastoma

15.4.1 Introduction

RB is the most common intraocular tumor of childhood. It occurs both in genetic (40 %) and sporadic forms (60 %). Advances in treatment, which includes recent adjuvant chemotherapy improved mortality of 100–90 % survival. There are reports showing 50 % cellular viability with retinocytoma like areas post chemotherapeutic regime (unpublished data). These features have raised questions about persistence

or propagation of CSCs in RB tumors. It is very useful to have better information of the heterogeneity of the tumor, in order to understand tumor progression, metastasis and this will be helpful in designing better therapy for future treatments.

15.4.2 Phenotypic Characteristics

RB is a tumor suppressor gene that regulates cell cycle progression and proliferation. If both alleles of the *RB* on chromosome 13 are mutated in a single retinal cell it results in retinoblastoma (Knudson 1971; Kyritsis et al. 1984).

15.4.3 Cell of Origin

In order to study the cell of origin, previously many investigators have explored the expression of neuronal, glial and other differentiated cell markers through immunohistochemical studies.

15.4.3.1 Neuronal Cell Markers

Neuron Specific Enolase (γ-γ NSE) a neuronal cell lineage marker is expressed in the normal retina in neurons, Muller cells, inner segments of cone cells, bipolar cells, outer and inner nuclear layers (Terenghi et al. 1984). NSE was also shown to be expressed in aqueous humor of retinoblastoma cases and the levels of expression range from 619 to 60,000 ng/ml (Kyritsis et al. 1984). In retinoblastoma eye balls, most of the small round tumor cells express NSE and the expression in rosettes is contradictory as some reports have shown to be weakly expressed, but others have shown strong expression (Molnar et al. 1984; Craft et al. 1985; Messmer et al. 1985). In tumors of neuro ectodermal origin like retinoblastoma, neuroblastoma and medulloblastoma expression of various enolases (i.e. α- α, α-γ and γ- γ) is seen. Based upon this and other markers investigators hypothesized that these all tumors have similar origin (Terenghi et al. 1984). N-CAM a neural cell adhesion molecule that appears to be involved in the regulation of adhesive interaction during neuronal differentiation was also shown to be expressed in all tumors (Antunez et al. 1991).

15.4.3.2 Glial Cell Markers

The mature glial cell markers glial fibrillary acidic protein and S-100 are frequently associated with retinoblastomas. Kivela et al reported that all undifferentiated cells contain both NSE and GFAP, where as differentiating neuronal and glial-like cells gradually lose one marker and selectively express a marker that correlates with their

morphology (Kivela and Virtanen 1986). In the normal retina, most of the glial cells in the ganglion cell layer and Muller cells express GFAP, S-100 and vimentin (Kivela 1986). It was reported that tumors with choroidal invasion lack expression of GFAP (Kivela 1986). In most of the reports it was shown that GFAP and S-100 was expressed in perivascular glial cells that were interpreted as reactive astrocytes. In Flexener – Wintersteiner rosettes and in the areas of photoreceptor like differentiation, glial cells reactive for LN-1, S-100, A_2B_5 and GFAP were demonstrated indicating differentiation along a glial direction in the more mature parts of retinoblastoma. GFAP and rhodopsin expression was negative in each of the ocular and pineal tumor in trilateral retinoblastoma. In one of six retinoblastoma tumors, rhodopsin is expressed in rosettes (Rodrigues et al. 1986).

15.4.3.3 Other Cell Markers

S-antigen expressed in normal photoreceptors shows several different patterns of S-antigen immunoreactivity in retinoblastomas indicating normal photoreceptor elements being incorporated into the growing tumor. In some cases this marker was associated with fleurettes and also isolated tumor cells and in ocular and pineal tumors of trilateral retinoblastoma (Nakajima et al. 1986). This marker is absent in undifferentiated retinoblastomas (Molnar et al. 1984). Expression of this antigen in retinoblastoma may be used to assess the degree of tumor differentiation. Another marker expressed in outer segments of photoreceptor elements is **Opsin**. This marker was shown to express in the fleurettes and rosettes of differentiated tumor. **Lactate dehydrogenase** is the isoenzyme which mostly concentrates in those regions where there is relatively high rate of metabolism. In the retinoblastoma tumors well differentiated areas was shown to be highly expressing this marker than undifferentiated tumors (Schroder 1987). **Vimentin** is expressed on muller cells in normal human retina, and on stromal cells, near the perivascular glial cells and reactive glial cells or muller cells in retinoblastomas. **Neurofilament protein** was shown to be expressed in axons of ganglion cells and in tumor-associated Flexener and Wintersteiner rosettes. Among the neurofilament triplet proteins NF68 and NF160, but not NF210 was expressed (Sawa et al. 1987). **Inter photoreceptor retinoid binding protein** was shown to be expressed in retinoblastoma tumors suggesting that the tumor also contains partial photoreceptor like differentiation. **Leu – 7** is a marker for neural bipolar cells and was normally expressed in well differentiated glial cells that were interpreted as reactive and not neoplastic cells (Perentes et al. 1987). If this marker is expressed, it was shown to be expressed in perivascular glial cells along with GFAP. Scattered cytoplasmic staining of **cGMP** and **cyclic GMP phosphodiesterase** was expressed in tumor cells of retinoblastoma. Certain **lectins** were shown to be expressed in retina and retinoblastoma tumor; this homology suggests biochemical as well as structural similarities between these tissues (Rodrigues et al. 1986). Onco foetal proteins like **α-Feto protein** and **carcino embryogenic protein** were shown to be present in the serum of retinoblastoma patients, but these proteins were not detected in the tumor cells.

The diversity of expression of several markers has, not surprisingly, led to disparate opinions regarding RB's cell of origin. Many believe it is a primitive multipotential cell, others suggest it is a cell capable of only bipotential differentiation (Photoreceptor and glial differentiation). Some investigators propose the tumor to be of strictly neuronal origin. Still others have proposed that the cell of origin is a differentiated photoreceptor or more specifically, a cone cell.

In the early days, Flexner and Wintersteiner hypothesized that photoreceptors might give rise to retinal tumors. This assumption was based on the morphology of rosettes observed in tumor samples (Cotran 1994). Early diagnosis of this tumor and availability of human samples is very rare; investigators have concentrated in creating animal models to study this disease. In the 1960s intraocular injection of adenovirus 12 produced retinal tumors in young rats, mice and baboons. These studies revealed that viral oncoproteins blocked Rb and its family members p107, p130 and many other proteins (Mukai and Kobayashi 1973; Ogawa et al. 1966, 1969). Later heritable retinal tumor mouse models were generated (LH-beta TAG mice) by expression of a viral oncogene simian virus 40 T-antigen. The tumors that were resulted by this method were comparable to human tumors in histological, ultra structural and immunohistochemical characteristics (Windle et al. 1990). Recently conditional knockout mouse models (Rb (–/–); p107 (+/–); p130 (–/–)) were generated and in these models differentiated horizontal interneurons re-entered the cell cycle, clonally expanded and formed metastatic retinoblastoma. In these models all the tumors formed were resembling differentiated and not showing the undifferentiated and moderately differentiated form of retinoblastoma in humans (Ajioka et al. 2007). In contrast to this study it was shown that human cone-specific signaling circuitry sensitizes to the oncogenic effects of *RB* mutations (Xu et al. 2009). It was also reported that p53 pathway was inactivated in retinoblastoma because of over expression of MDMX gene in this tumor and if this MDMX gene is targeted using nutlin-b it resulted in the death of tumor cells (Laurie et al. 2006).

In addition to the mouse models of retinoblastoma there are two very well characterized human retinoblastoma cell lines (Y79 and WERI-RB27) obtained from the patients diagnosed for retinoblastoma (Reid et al. 1974; Sery et al. 1990). These cell lines provide close models to the human cases for experimentation. Main difference between human and mouse models of RB is that mouse RB requires inactivation of *RB* product and also its family members but it is not the case for humans where RB inactivation is sufficient to produce tumors (Pacal and Bremner 2006).

15.4.4 Evidences of Stem Cells in Retinoblastoma

The study on retinoblastoma has changed fundamentally the understanding of tumor biology, primarily genetics, mode of inheritance and the means to survival. Recent studies have shown the expression of drug resistance markers like multi drug resistant p-glycoprotein and lung resistance protein has provided the evidence of stem cells in RB (Krishnakumar et al. 2004). ABCG2 a cell surface marker has been used

to characterize stem cells. It has been shown that cells expressing this marker are present within a pool of Hoechst 33342 low cells and it was shown for the first time in hematopoietic populations, and latter in skeletal muscle, mammary gland, lung and developing retina etc. (Bhattacharya et al. 2003; Scharenberg et al. 2002; Summer et al. 2003; Welm et al. 2002). Recent studies have shown the presence of cells expressing ABCG2 positive and Hoechst33342 low cells in mouse RB and human RB cell lines ranging from 0.1 to 0.4 % of the total population (Setoguchi et al. 2004). In addition to these markers immunoreactivity to other markers like ALDH1, oct3/4, Nanog, MCM2 and sca-1 was detected in mouse models (Seigel et al. 2007). Expression of MCM2 (mini chromosome maintenance gene) have reported in large number of retrospective samples in which Mohan et al. have shown MCM2 and ABCG2 in more than 50 % of RB tumor samples examined (Mohan et al. 2006). The expression of these markers has correlated with highly invasive tumors. Recently it was shown in human tumors that retinoblastoma cell MDM2 expression was regulated by the cone-specific RXR gamma transcription factor, human RXR gamma consensus binding site and cone specific thyroid hormone receptor-beta$_2$. It was also shown that CRX^+ cells are Rb^- and were the neoplastic components. Results of this study provided support for a cone precursor cell of origin in retinoblastoma (Xu et al. 2009). Another recent study have shown that Wnt signaling activator LiCl increased the number of stem-like cells in retinoblastoma (Silva et al. 2010).

15.4.5 Hierarchy of Stem Cells in Retinoblastoma Tumors

Recent report of ours has shown evidence towards hierarchical model of origin in retinoblastoma (Balla et al. 2009). We found two different subpopulations based on scatter properties and marker expression. FSC^{lo}/SSC^{lo} subpopulation appeared to be more primitive, since they expressed stem cell (CD44) and retinal progenitor markers ($PROX_1$, SYX_1a) in addition to the lower percentage of differentiated markers (CD90, CD133, NSE). The other subpopulation FSC^{hi}/SSC^{lo} showed a higher percentage of differentiated markers and low expression of retinal progenitor cell markers (Figs. 15.3 and 15.4). These results have suggested the hierarchy and heterogeneity of cells in retinoblastoma tumors.

15.5 Treatment Implications: Understanding the Mechanisms of Resistance

Illustration of the CSC hypothesis in diverse solid tumors has provided new insights into new generation therapies for cancer. The main obstacle towards effective treatment remains the failure of current therapy in eradicating all tumor cells to prevent

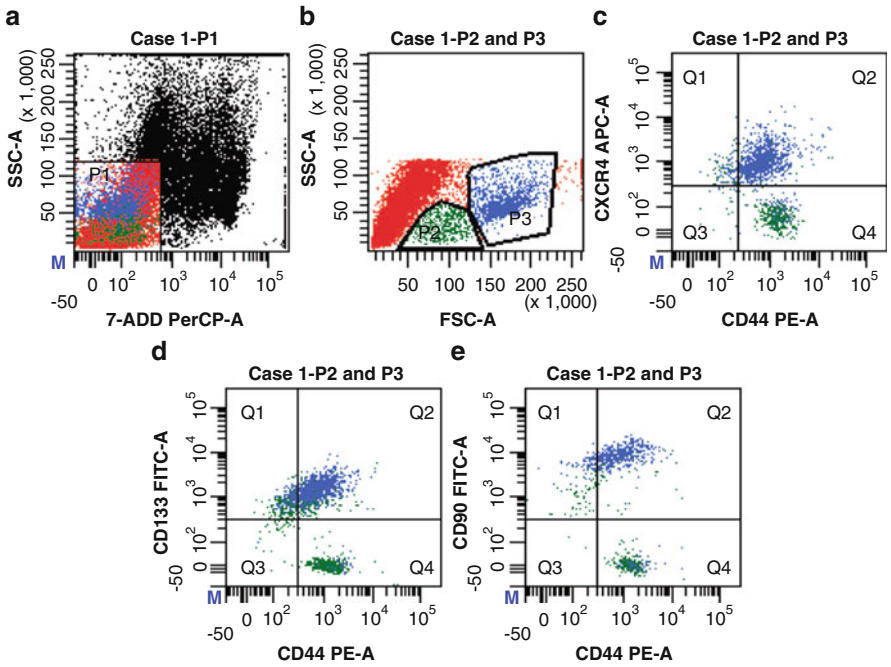


Fig. 15.3 Differential expression of stem and differentiated cell markers in Rb cells. *Panel a*, – the P1 gate in this plot shows cells which were negative for 7-AAD stain. *Panel b*, shows P1 selected cells in FSC vs SSC plot. P2 gate shows the FSC^{lo} SSC^{lo} sub-population (shown in *green*) and P3 gate shows the sub-population FSC^{hi} SSC^{lo} (shown in *blue*). Population shown in red was eliminated from analysis. *Panels c–e* show the population of cells which were CD44 positive and negative for CXCR4, CD133 and CD90 (represented in Q4 quadrant)

disease recurrence, thereby affecting long-term survival. Traditionally, drug therapies have been developed based on the ability of these agents to cause tumor regression in animal models. Since it has now been shown that a majority of the cancer cells within the tumor are non-tumorigenic, therapies directed against these cells would initially cause tumor regression, followed by recurrence as a consequence of persisting CSCs. Thus the goal of cancer therapy should be to generate drugs that target CSCs. However, such therapeutic strategies against CSC fractions require elucidation of key mechanisms by which these cells resists existing therapies.

15.5.1 Quiescence

In view of the fact that majority of cancer therapies are designed against rapidly proliferating cells, quiescent populations would automatically be shielded from therapeutic attack. Thereby quiescence, a defining trait for stem cells has been

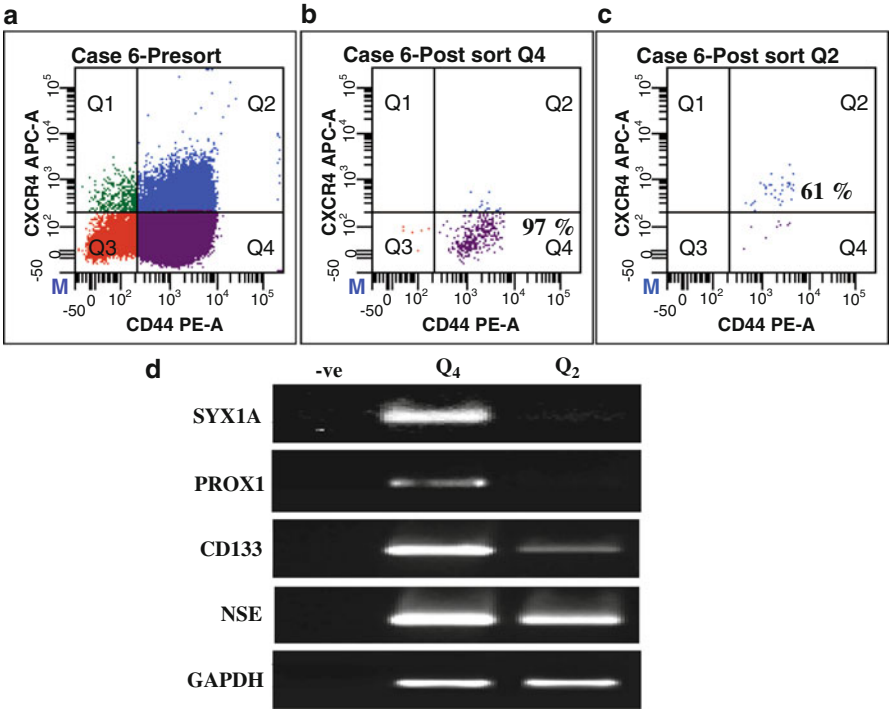


Fig. 15.4 Total RNA was extracted from the cell populations in the Q2 and Q4 quadrants after FACS sorting of Rb cells from the tumor of case 6. *Panel a*, shows the presort analysis of CD44 and CXCR4 expression *Panel b*, shows the post sort efficiency of Q4 (97 %) *Panel c*, shows the post sort efficiency of Q2 (61 %) *Panel d*, shows the RT-PCR analysis for human Syntaxin1A, PROX1, CD133, NSE and GAPDH

speculated extensively as mechanism of therapeutic resistance since the origin of CSC paradigm. However, experimental proof for the same was lacking until recently, when through long-term label retention potential, we demonstrated CSCs to remain quiescent within tumor niche. Long-term label retention that exploits relatively quiescent nature of stem cells is a classical approach for identification of tissue specific stem cells (Blanpain et al. 2004; Tumbar et al. 2004; Yue et al. 2005). Using the membrane labeling vital dyes PKH26/67, we identified CSCs in experimentally generated ovarian tumors as marked, *in vivo* residing label retaining cells (LRCs) within the tumor niche (Fig. 15.5). Therapeutic refractoriness demonstrated by the long-term label retaining CSCs coupled with the potential to regenerate the tumors post therapeutic regression substantiated their role in tumor dormancy (Kusumbe and Bapat 2009) In corroboration with our results, a recent study has demonstrated the label retention potential of CSCs from breast cancer (Pece et al. 2010). These initial studies exploiting label retention for claiming CSC quiescence provide robust models for further investigations to identify mechanisms involved in maintenance of quiescent state.

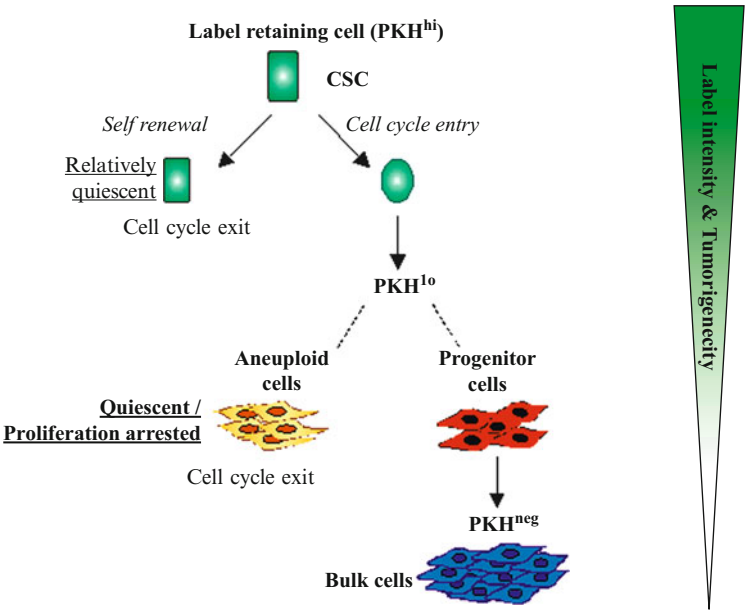


Fig. 15.5 Schematic representing the label retention based approach for the identification of the cell types contributing to the tumor dormancy. CSCs demonstrated long-term label retention and could be isolated as LRCs. At the same time aneuploid cells had low levels of the label intensity and were trapped within the PKH^{lo} compartment as a consequence of their quiescence/proliferation arrest while the bulk tumor cells constituted the PKH^{neg} compartment due to the proliferation directed complete loss of the label

15.5.2 Niche

Successful *in situ* identification of CSCs as LRCs prompted us to identify LRCs within the cell lines. However in contrast to developing tumors, cell lines seemed to be devoid of LRCs (Kusumbe and Bapat 2009). Such failure to identify quiescent cells in culture suggests that analogous to the NSCs, maintenance of CSCs apart from intrinsic factors also substantially depends on external signals. These external signals collectively make up the microenvironment or niche (classically defined as an interactive structural unit, organized to facilitate cell-fate decisions in a proper spatiotemporal manner) that has been implicated to play a crucial role in maintenance of the CSC compartment (Moore and Lemischka 2006). Evidence for this notion came from a recent study that suggests involvement of vascular niches in maintenance of brain CSC compartment. Specifically, the study illustrates vascular niches within the brain tumors are aberrant and drives CSC self-renewal and proliferation that is in contrast to the niches found in the normal tissue that usually control the stem cell function (Calabrese et al. 2007).

Microenvironment mediated drug resistance envisaged and studied since a long time is a form of *de novo* resistance that protects the tumor cells from the affects of therapies (Mark et al. 2009). Above preliminary investigations in field imply that even CSCs may depend on their microenvironment/niche to resist the therapeutic regimes.

If indeed vascular niches are required for self-renewal and proliferation of CSCs as discussed above then development of such niches within tumor tissue would not only contribute tumor vasculature but also ensure long-term maintenance of CSCs in these protective niches thereby augmenting disease progression. Support for this notion comes from our finding that CSCs demonstrate an active recruitment of primitive endothelial stem cells that are capable of establishing an entire tumor endothelial hierarchy. Further the recruited endothelial stem cells also demonstrate an intimate physical association with CSCs; thereby claiming the instructive role of niche in CSC maintenance (Kusumbe et al. 2009). These recruited endothelial stem cells initiated and established the entire endothelial hierarchy thus contributing to tumor vasculature which augmented tumor growth and progression. These findings also support the previous studies proposing that the reciprocal interaction between the tumor cells and microenvironment controls the switch between proliferation and quiescence (Aguirre-Ghiso 2007; White et al. 2004).

15.5.3 Stress Induced CSC Enrichment

Above evidences suggest that oncogenic signaling might not always be dominant and that other programs (such as stem cell quiescence and microenvironment/niche restrictions) might overcome oncogenic signals, thereby governing CSC function and behavior. Application of treatment regime has been known to generate stress within the local tumor environment that leads to activation of stress pathways in CSCs enabling their persistence and accumulation (White et al. 2004). One such example is stress induced up-regulation of transcription factors Snail and Slug that not only aids cell survival but also leads to acquisition of chemoresistance and radioresistance (Kurrey et al. 2009). The fact that CSCs specifically exploit such pathways is affirmed by frequent documentation of their enrichment under various stress conditions including chemotherapy, radiotherapy, hypoxia, serum depletion etc. Further stress induced disease progression that mainly involves metastasis is also a frequent observation in cancer biology. To metastasize, cancer cells must detach from neighboring epithelial cells and adopt a mesenchymal phenotype i.e. cells must undergo epithelial to mesenchymal transition (EMT). Research in the field has revealed a number of pleiotropically acting transcription factors including Snail, Slug and Twist that play critical roles in EMTs not only during embryogenesis but also during tumorigenesis. Recent investigations illustrates that these transcription factors on upregulation under

stress, besides orchestrating EMT and aiding therapeutic resistance also mediate acquisition of stem cell characteristics (Kurrey et al. 2009; Mani et al. 2008). Thus orchestration of transcription machinery leading to cooperative modulation of gene expression seems to be an important mechanism for achieving therapeutic resistance through enrichment of CSC under stress.

15.5.4 Genetic Instability

Cancer is classically recognized as a disease of clonal evolution. Aneuploid cells, due to their genetic instability, possess adaptive growth advantages and hence are thought to be crucial determinants of caner recalcitrance (Sieber et al. 2003; Weaver and Cleveland 2007) Consistent with this, we identified that apart from CSCs that contribute to tumor dormancy, aneuploid population too constitute major determinants of cancer dormancy. Aneuploid cells that are proliferation arrested/quiescent under steady state undergo selective pressure/stress acquired proliferation potency induced by chemotherapeutic exposure. These findings suggests that the existing pool of CSCs constantly generates a highly aneuploid progeny that stays proliferation arrested under the no/minimal stress conditions hence constituting just a dormant subset within the tumors. However on exposure to a stress condition or selective pressure (e.g. drug shock) these cells are recruited into cell cycle. Persistence of such genetically unstable dormant aneuploid cells packaged with remarkable adaptive and selective capacities has profound clinical implications for neoplastic progression and cancer therapy. Such stress induced acquisition of proliferation potency along with additional adaptive capacities inherent to these cells would lead to an emergence of a new CSC pool. The new CSC pool may dominate and take over the existing CSC pool or both of them may be retained concurrently thereby accounting for the CSC heterogeneity that has been documented in many recent reports (Visvader and Lindeman 2008). This genetic instability leading to CSC evolution further explains the inefficacy in obtaining complete eradication of these cells during the treatment regimes.

15.6 Future Directions and Perspectives

All efforts towards identification and characterization of the CSCs should be ultimately focused to create a conducive framework that facilitates designing of targeted therapeutic strategies against CSC population. This is particularly essential as the CSC paradigm implicates that for long-term disease-free survival, CSC population should be completely eradicated. Since CSCs share several characteristics with NSCs most critical aspect underlying the development of

efficient therapies would be selective targeting of the CSCs sparing their normal counterparts. A recent study demonstrating successful specific depletion of only leukemia-initiating cells has provided an initial support for this notion (Yilmaz et al. 2006). Such investigation could be achieved in the hematopoietic system due to the availability of established xenotransplant systems in which the potential of NSCs for self-renewal, multi-lineage differentiation, and proliferation can be evaluated. This allowed *in vivo* assessment of the toxicity of drug treatment to HSCs apart from the CSCs. However such characterization assays for normal stem/progenitor population from most of the non-hematopoetic tissues are lacking. Second major obstacle towards development of targeted therapy is the overlap between the currently used NSC and CSC markers. Identification of the markers exclusively expressed by CSCs which would be absent on their normal counterparts, will enable specific tracking of CSCs within the bulk tumor on a background of normal cells following a therapeutic exposure. Such an experimental set up is not only imperative to analyze toxicity of drug specifically on the NSC population but can also be exploited for developing specific targeting strategies.

Apart from the technical drawbacks several intrinsic peculiarities of CSCs presents a major challenge in designing effective therapeutic strategies. Such intrinsic factors include:

(i) Drug resistance driven by several differential mechanisms acting as a cohort (Kurrey et al. 2009; Eylerand and Rich 2008);
(ii) Stemness features like quiescence and self-renewal contributing to tumor dormancy and subsequent relapse (Reya et al. 2001; Kurrey et al. 2009);
(iii) Genetic instability and epigenetic variability (Visvader and Lindeman 2008) ultimately thriving CSC heterogeneity and evolution that along with providing enormous survival benefits to this population, also makes the system complex for therapeutic targeting.

On this background, recent studies illustrating dependency of CSCs for their maintenance on the external signals (niche) suggests niche ablation as a powerful therapeutic strategy (Sneddon and Werb 2007). Again feasibility of this approach depends to a large extent on degree of similarity between normal and CSC niches. If identical factors/signals drive survival and proliferation in both the niches, then niche ablation will also distress NSC pools. Recent observations illustrating the existence of a modified and aberrant niche to support the brain CSC compartment, strengthens the applicability of niche ablation as a therapeutic approach to ultimately bring about complete exhaustion of CSC compartment. However, such investigations detailing the CSC niche are still at the stage of infancy (Fig. 15.6). A continuing identification of additional mechanisms and signals underlying the phenomenon, specifically key distinguishing features between normal and CSC niches will be critical for developing therapeutic strategies adept for direct and specific abolishment of only CSC niche without or with minimal toxicity to NSC niche.

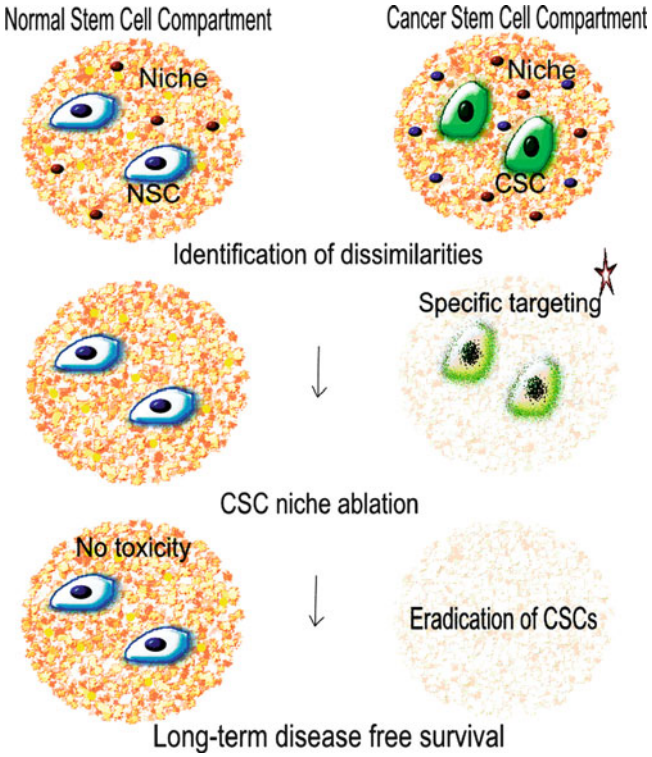


Fig. 15.6 Schematic illustrating the specific targeting of the CSCs may be possible through niche ablation

Acknowledgements We thank Dr. G.C. Mishra, Director, National Center for Cell Science (Pune, India) for encouragement and support. We thank Dr. Santosh Honavar and team for providing clinical samples for Retinoblastoma work. We also acknowledge the Association for Research in Vision and Ophthalmology, the copyright holder of Figs. 15.3 and 15.4 for permitting use of these figures

References

Aguirre-Ghiso J (2007) Models, mechanisms and clinical evidence for cancer dormancy. Nat Rev Cancer 7:834–846

Ajioka I, Martins R, Bayazitov I et al (2007) Differentiated horizontal interneurons clonally expand to form metastatic retinoblastoma in mice. Cell 131:378–390

Al-Hajj M, Wicha M, Benito-Hernandez A et al (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100:3983–3988

Antunez J, Couce M, Fraga M et al (1991) Immunohistochemical demonstration of neuronal and astrocytic markers and oncofoetal antigens in retinoblastomas. Histol Histopathol 6:241–246

Balla M, Vemuganti G, Kannabiran C et al (2009) Phenotypic characterization of retinoblastoma for the presence of putative cancer stem-like cell markers by flow cytometry. Invest Ophthalmol Vis Sci 50:1506–1514

Bapat S (ed) (2009) Cancer stem cells. Wiley, Hoboken
Bapat S, Mali A, Koppikar C et al (2005) Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 65:3025–3029
Bhattacharya S, Jackson J, Das A et al (2003) Direct identification and enrichment of retinal stem cells/progenitors by hoechst dye efflux assay. Invest Ophthalmol Vis Sci 44:2764–2773
Blanpain C, Lowry W, Geoghegan A et al (2004) Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 118:635–648
Bonnet D, Dick J (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3:730–737
Calabrese C, Poppleton H, Twala K (2007) Perivascular niche for brain tumor stem cells. Cancer Cell 11:69–82
Challen G, Little M (2006) A side order of stem cells: the sp phenotype. Stem Cells 24:3–12
Chiba T, Kita K, Zheng Y et al (2006) Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. Hepatology 44:240–251
Cobaleda C, Gutierrez-Cianca N, Perez-Losada J et al (2000) A primitive hematopoietic cell is the target for the leukemic transformation in human philadelphia-positive acute lymphoblastic leukemia. Blood 95:1007–1013
Collins A, Berry P, Hyde C, Stower M et al (2005) Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65:10946–10951
Cotran SR (1994) Pathologic basis of disease, 5th edn. WB Saunders Company, Philadelphia
Courtenay V, Mills J (1978) An in vitro colony assay for human tumours grown in immune-suppressed mice and treated in vivo with cytotoxic agents. Br J Cancer 37:261–268
Courtenay V, Selby P, Smith I (1978) Growth of human tumor cell colonies from biopsies using two soft-agar techniques. Br J Cancer 38:77–81
Craft J, Sang D, Dryja T et al (1985) Glial cell component in retinoblastoma. Exp Eye Res 40:647–659
Decraene C, Benchaouir R, Dillies M et al (2005) Global transcriptional characterization of sp and mp cells from the myogenic c2c12 cell line: effect of fgf6. Physiol Genomics 23:132–149
Duncan A, Rattis F, DiMascio L et al (2005) Integration of notch and wnt signaling in hematopoietic stem cell maintenance. Nat Immunol 6:314–322
Eylerand C, Rich J (2008) Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. J Clin Oncol 26(17):2839–2845
Fearon E, Hamilton S, Vogelstein B (1987) Clonal analysis of human colorectal tumors. Science 238:193–197
Fialkow P (1976) Clonal origin of human tumors. Biochim Biophys Acta 458:283–321
Goodell M, Brose K, Paradis G et al (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183:1797–1806
Grichnik J, Burch J, Schulteis R et al (2006) Melanoma, a tumor based on a mutant stem cell? J Invest Dermatol 126:142–153
Hamburger A, Salmon S (1977) Primary bioassay of human tumor stem cells. Science 197:461
Hewitt H (1958) Studies of the dissemination and quantitative transplantation of a lymphocytic leukaemia of CBA mice. Br J Cancer 12(3):378–401
Hirschmann-Jax C, Foster A, Wulf G et al (2004) A distinct “Side population” of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci USA 101:14228–14233
Hopfer O, Zwahlen D, Fey M et al (2005) The notch pathway in ovarian carcinomas and adenomas. Br J Cancer 93:709–718
Jordan C, Guzman M, Noble M (2006) Cancer stem cells. N Engl J Med 355:1253–1261
Katano M (2005) Hedgehog signaling pathway as a therapeutic target in breast cancer. Cancer Lett 227:99–104
Kim C, Jackson E, Woolfenden A et al (2005) Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 121:823–835
Kiran V, Kannabiran C, Chakravarthi K et al (2003) Mutational screening of the rb1 gene in indian patients with retinoblastoma reveals eight novel and several recurrent mutations. Hum Mutat 22:339

Kivela T (1986) S-100 protein in retinoblastoma revisited. An immunohistochemical study. Acta Ophthalmol (Copenh) 64:664–673
Kivela T, Virtanen I (1986) Intermediate filaments in the human retina and retinoblastoma. An immunohistochemical study of vimentin, glial fibrillary acidic protein, and neurofilaments. Invest Ophthalmol Vis Sci 27:1075–1084
Knudson A (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA 68:820–823
Kolligs F, Bommer G, Goke B (2002) Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis. Digestion 66:131–144
Kondo T, Setoguchi T, Taga T (2004) Persistence of a small subpopulation of cancer stem-like cells in the c6 glioma cell line. Proc Natl Acad Sci USA 101:781–786
Krishnakumar S, Mallikarjuna K, Desai N et al (2004) Multidrug resistant proteins: P-glycoprotein and lung resistance protein expression in retinoblastoma. Br J Ophthalmol 88:1521–1526
Krivtsov A, Twomey D, Feng Z (2006) Transformation from committed progenitor to leukaemia stem cell initiated by mll-af9. Nature 442:818–822
Kruh G (2003) Introduction to resistance to anticancer agents. Oncogene 22:7262–7264
Kurrey N, Jalgaonkar S, Joglekar A et al (2009) Snail and slug mediate radio- and chemo-resistance by antagonizing p53-mediated apoptosis and acquiring a stem-like phenotype in ovarian cancer cells. Stem Cells 27(9):2059–2068
Kusumbe A, Bapat S (2009) Cancer stem cells and aneuploid populations within developing tumors are the major determinants of tumor dormancy. Cancer Res 69(24):9245–9253
Kusumbe A, Mali A, Bapat S (2009) CD133-expressing stem cells associated with ovarian metastases establish an endothelial hierarchy and contribute to tumor vasculature. Stem Cells 27:498–508
Kyritsis A, Tsokos M, Triche T et al (1984) Retinoblastoma – origin from a primitive neuroectodermal cell? Nature 307:471–473
Lapidot T, Sirard C, Vormoor J et al (1994) A cell initiating human acute myeloid leukaemia after transplantation into scid mice. Nature 367:645–648
Larderet G, Fortunel N, Vaigot P et al (2006) Human side population keratinocytes exhibit long-term proliferative potential and a specific gene expression profile and can form a pluristratified epidermis. Stem Cells 24:965–974
Laurie N, Donovan S, Shih C et al (2006) Inactivation of the p53 pathway in retinoblastoma. Nature 444:61–66
Li C, Heidt D, Dalerba P et al (2007) Identification of pancreatic cancer stem cells. Cancer Res 67:1030–1037
Mani S, Guo W, Liao M et al (2008) The epithelialmesenchymal transition generates cells with properties of stem cells. Cell 133:704–715
Mark B, Meads R, Dalton G (2009) Environment-mediated drug resistance: a major contributor to minimal residual disease. Nat Rev Cancer 9:665–667
Matsui W, Huff C, Wang Q et al (2004) Characterization of clonogenic multiple myeloma cells. Blood 103:2332–2336
Messmer E, Font R, Kirkpatrick J et al (1985) Immunohistochemical demonstration of neuronal and astrocytic differentiation in retinoblastoma. Ophthalmology 92:167–173
Mohan A, Kandalam M, Ramkumar H et al (2006) Stem cell markers: Abcg2 and mcm2 expression in retinoblastoma. Br J Ophthalmol 90:889–893
Molnar M, Stefansson K, Marton L et al (1984) Immunohistochemistry of retinoblastomas in humans. Am J Ophthalmol 97:301–307
Moore M (1991) Clinical implications of positive and negative hematopoietic stem cell regulators. Blood 78:1–19
Moore K, Lemischka I (2006) Stem cells and their niches. Science 311:1880–1885
Mukai N, Kobayashi S (1973) Human adenovirus-induced medulloepitheliomatous neoplasms in Sprague–Dawley rats. Am J Pathol 73:671–690
Nakajima T, Kato K, Kaneko A et al (1986) High concentrations of enolase, alpha- and gamma-subunits, in the aqueous humor in cases of retinoblastoma. Am J Ophthalmol 101:102–106

Nowell P (1976) The clonal evolution of tumor cell populations. Science 194:23–28
O'Brien C, Pollett A, Gallinger S et al (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445:106–110
Ogawa K, Tsutsumi A, Iwata K et al (1966) Histogenesis of malignant neoplasm induced by adenovirus type 12. Gann 57:43–52
Ogawa K, Hamaya K, Fujii Y et al (1969) Tumor induction by adenovirus type 12 and its target cells in the central nervous system. Gann 60:383–392
Ogawa M, Bergsagel D, McCulloch E (1973) Chemotherapy of mouse myeloma: quantitative cell cultures predictive of response in vivo. Blood 41:7–15
Pacal M, Bremner R (2006) Insights from animal models on the origins and progression of retinoblastoma. Curr Mol Med 6:759–781
Park C, Amare M, Savin M (1980) Prediction of chemotherapy response in human leukemia using an in vitro chemotherapy sensitivity test on the leukemic colony-forming cells. Blood 55:595–601
Pece S, Tosoni D, Confalonieri S et al (2010) Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell 140(1):62–73
Peeters S, van der Kolk D, de Haan G et al (2006) Selective expression of cholesterol metabolism genes in normal $CD34^{+}CD38^{-}$ cells with a heterogeneous expression pattern in aml cells. Exp Hematol 34:622–630
Perentes E, Herbort C, Rubinstein L et al (1987) Immunohistochemical characterization of human retinoblastomas in situ with multiple markers. Am J Ophthalmol 103:647–658
Prince M, Sivanandan R, Kaczorowski A et al (2007) Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci USA 104:973–978
Puck T, Marcus P (1956) Action of x-rays on mammalian cells. J Exp Med 103:653–666
Puck T, Marcus P, Cieciura S (1956) Clonal growth of mammalian cells in vitro; growth characteristics of colonies from single Hela cells with and without a feeder layer. J Exp Med 103:273–283
Rask K, Nilsson A, Brannstrom M et al (2003) Wnt-signalling pathway in ovarian epithelial tumours: increased expression of beta-catenin and gsk3beta. Br J Cancer 89:1298–1304
Reid T, Albert D, Rabson A et al (1974) Characteristics of an established cell line of retinoblastoma. J Natl Cancer Inst 53:347–360
Reya T, Morrison S, Clarke M et al (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105–111
Ricci-Vitiani L, Lombardi D, Pilozzi E et al (2007) Identification and expansion of human colon-cancer-initiating cells. Nature 445:111–115
Rodrigues M, Wilson M, Wiggert B et al (1986) Retinoblastoma. A clinical, immunohistochemical, and electron microscopic case report. Ophthalmology 93:1010–1015
Sanchez P, Clement V, Ruizi Altaba A (2005) Therapeutic targeting of the hedgehog-gli pathway in prostate cancer. Cancer Res 65:2990–2992
Sawa H, Takeshita I, Kuramitsu M et al (1987) Immunohistochemistry of retinoblastomas. J Neurooncol 5:351–355
Scharenberg C, Harkey M, Torok-Storb B (2002) The abcg2 transporter is an efficient hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood 99:507–512
Schroder H (1987) Immunohistochemical demonstration of glial markers in retinoblastomas. Virchows Arch A Pathol Anat Histopathol 411:67–72
Seigel G, Hackam A, Ganguly A et al (2007) Human embryonic and neuronal stem cell markers in retinoblastoma. Mol Vis 13:823–832
Sery T, Lee E, Lee W et al (1990) Characteristics of two new retinoblastoma cell lines: Weri-rb24 and weri-rb27. J Pediatr Ophthalmol Strabismus 27:212–217
Setoguchi T, Taga T, Kondo T et al (2004) Cancer stem cells persist in many cancer cell lines. Cell Cycle 3:414–415
Sieber O, Heinimann K, Tomlinson I (2003) Genomic instability – the engine of tumorigenesis? Nat Rev Cancer 3:701–708

Silva A, Yi H, Hayes S et al (2010) Lithium chloride regulates the proliferation of stem-like cells in retinoblastoma cell lines: a potential role for the canonical wnt signaling pathway. Mol Vis 16:36–45

Singh S, Hawkins C, Clarke I et al (2004) Identification of human brain tumour initiating cells. Nature 432:396–401

Sneddon J, Werb Z (2007) Location, location, location: the cancer stem cell niche. Cell Stem Cell 1:607–611

Southam C, Brunschwig A (1960) A quantitative studies of autotransplantation of human cancer. Cancer 14:971–978

Suetsugu A, Nagaki M, Aoki H et al (2006) Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. Biochem Biophys Res Commun 351:820–824

Summer R, Kotton D, Sun X et al (2003) Side population cells and bcrp1 expression in lung. Am J Physiol Lung Cell Mol Physiol 285:L97–L104

Szotek P, Pieretti-Vanmarcke R, Masiakos P et al (2006) Ovarian cancer side population defines cells with stem cell-like characteristics and mullerian inhibiting substance responsiveness. Proc Natl Acad Sci USA 103:11154–11159

Terenghi G, Polak J, Ballesta J et al (1984) Immunocytochemistry of neuronal and glial markers in retinoblastoma. Virchows Arch A Pathol Anat Histopathol 404:61–73

Tumbar T, Guasch G, Greco V et al (2004) Defining the epithelial stem cell niche in skin. Science 303:359–363

Umemoto T, Yamato M, Shiratsuchi Y et al (2006) Expression of integrin beta3 is correlated to the properties of quiescent hemopoietic stem cells possessing the side population phenotype. J Immunol 177:7733–7739

Visvader J, Lindeman G (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8:755–768

Wani A, Sharma N, Shouche Y et al (2006) Nuclear-mitochondrial genomic profiling reveals a pattern of evolution in epithelial ovarian tumor stem cells. Oncogene 25:6336–6344

Weaver B, Cleveland D (2007) Aneuploidy: instigator and inhibitor of tumorigenesis. Cancer Res 67:10103–10105

Welm B, Tepera S, Venezia T et al (2002) Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol 245:42–56

White D, Kurpios N, Zuo D et al (2004) Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. Cancer Cell 6:159–170

Wilson A, Radtke F (2006) Multiple functions of notch signaling in self-renewing organs and cancer. FEBS Lett 580:2860–2868

Windle J, Albert D, O'Brien J et al (1990) Retinoblastoma in transgenic mice. Nature 343:665–669

Xu X, Fang Y, Lee T et al (2009) Retinoblastoma has properties of a cone precursor tumor and depends upon cone-specific mdm2 signaling. Cell 137:1018–1031

Yilmaz O, Valdez R, Theisen B et al (2006) Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature 441:475–482

Yue Z, Jiang T, Widelitz R et al (2005) Mapping stem cell activities in the feather follicle. Nature 438:1026–1029

Zagzag D, Krishnamachary B, Yee H et al (2005) Stromal cell-derived factor-1alpha and cxcr4 expression in hemangioblastoma and clear cell-renal cell carcinoma: Von hippel-lindau loss-of-function induces expression of a ligand and its receptor. Cancer Res 65:6178–6188

Chapter 16
Mesenchymal Stem Cells – An Oversimplified Nomenclature for Extremely Heterogeneous Progenitors

Patrick Wuchter, Wolfgang Wagner, and Anthony D. Ho

Abstract Mesenchymal stem cells (MSC) are plastic-adherent fibroblast-like cells that can readily be isolated from various tissues and expanded in vitro. *Per definitionem*, they are able to differentiate into bone, cartilage and adipose tissue. In the last 15 years, a huge number of different preparative protocols have been developed to yield MSC-like cell lines from starting materials as diverse as bone marrow, fat tissue, umbilical cord blood and peripheral blood. However, these protocols as well as the resulting cell populations are heterogeneous. Furthermore, the composition of the cell products and their differentiation potential changes in the course of long-term culture expansion. There is an urgent need for the development of molecular markers and universal criteria for quality control of the starting cell populations as well as for the cell products after expansion. Nevertheless, MSC have already found their way into a huge number of clinical studies addressing a broad variety of diseases. Even though there is no convincing evidence that MSC are involved in the process of tissue repair by transdifferentiation, they probably contribute to the repair process by immunomodulatory effects and interaction with other cell types.

16.1 Introduction

Almost 40 years ago, in the early 1970s, Friedenstein et al. described discrete fibroblast-like colonies in monolayer cultures of bone marrow, spleen and thymus that could be easily maintained under culture conditions and that demonstrated

P. Wuchter, M.D. • A.D. Ho, M.D., FRCPC (✉)
Department of Internal Medicine V, Heidelberg University,
Im Neuenheimer Feld 410, 69120 Heidelberg, Germany
e-mail: anthony_dick.ho@urz.uni-heidelberg.de

W. Wagner, M.D., Ph.D.
Institute for Biomedical Engineering, University of Aachen Medical School,
Pauwelsstrasse 20, 52074 Aachen, Germany

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_16,

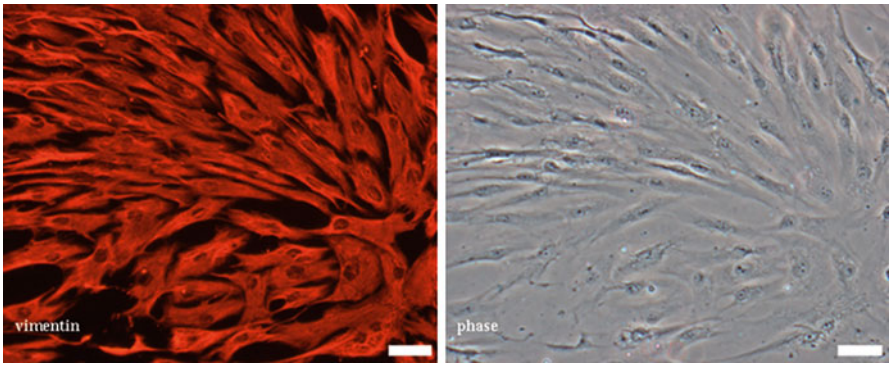


Fig. 16.1 MSC express vimentin, an intermediate filament typical for cells of mesenchymal origin. The cells are showing a fibroblastoid morphology. Immunofluorescent staining (*left*) and phase contrast (*right*) (scale bar 150 μm)

differentiation characteristics in vitro as well as in vivo upon their re-transplantation (Friedenstein et al. 1968, 1974). The term "mesenchymal stem cells" (MSC) however, has been made popular in the early 1990s by Caplan (1991). Caplan and others used periosteal cells from young chicken, which were transplanted into athymic mice and demonstrated their osteo-chondrogenic differentiation potential (Horwitz and Keating 2000; Nakahara et al. 1990). Today, the term "MSC" is commonly applied to plastic-adherent cell preparations isolated from bone marrow or other tissues that are able to differentiate into bone, cartilage and adipose tissue under specific conditions (see Fig. 16.1). Although there exist no specific markers for these cells, they are usually positive for several antigens such as CD73, CD90, CD105 and lack expression of hematopoietic antigens (Dominici et al. 2006; Wagner et al. 2005a).

The multilineage differentiation potential of MSC is still under debate. It is commonly accepted that a rare mesenchymal progenitor cell population is present in bone marrow, that possesses differentiation potential towards different cell lineages. However, mounting evidence indicates that the described adherent cell populations are highly heterogeneous and actually consist of several subpopulations, which gradually overgrow under certain culture conditions and might mimick the phenomenon of differentiation. Therefore, these cells might not fulfill all the criteria to be named "stem cells" and should therefore be named "mesenchymal stromal cells" (Horwitz and Keating 2000). Consequently, the acronym "MSC" stays the same, whereas the term "mesenchymal stem cells" should only be reserved for cells that meet specified criteria for stem cells, i.e. unlimited self-renewal capacity. Alternatively they have been named "multipotent mesenchymal stromal cells" or "multipotent stromal cells" to indicate the multipotent differentiation capacity of these cell preparations (Horwitz et al. 2005). The term "mesenchymal progenitor cell" (MPC) has also been used in analogy to the hematopoietic system where hematopoietic stem cells (HSC) are comprised within the CD34-positive cell fraction of hematopoietic progenitor cells (HPC) (Johnstone et al. 1998). Numerous

authors have described protocols for the isolation and cultivation of MSC from tissues other than the bone marrow, mainly from umbilical cord blood and adipose tissue. Beyond that, several subsets of MSC with possibly higher differentiation potential have been described. The terms "multipotent adult progenitor cells" (MAPC) (Jiang et al. 2002a), "unrestricted somatic stem cells" (USSC) (Kogler et al. 2004) and "very small embryonic-like" (VSEL) stem cells (Kucia et al. 2006a) have been applied to these subsets.

16.2 Derivation of MSC

The source and the property of MSC preparations from different laboratories vary significantly and the resulting cell products are highly heterogeneous. The lack of standardization considerably hampers the comparability of results among different research groups (Wagner and Ho 2007; Bieback et al. 2012).

We and others have demonstrated that slight differences of the culture conditions could favor the expansion of certain subsets and might contribute to genetic instability. Based on the morphology of MSC preparations, three distinct cell types could be distinguished: spindle-shaped cells, large flat cells and small round-cell subpopulations (Colter et al. 2001; Horn et al. 2008; Schallmoser et al. 2009). MSC cultures are continuously unstable and can give rise to individual cells – and subsequently cell colonies – producing, for example, smooth muscle-typical α-actin filaments and myofilaments containing cardiac α-actin (Ho et al. 2008). Even other cells in the culture start to form sub-lines positive for cytokeratin filaments. An entirely different program may be characteristic for other MSC, which begin to synthesize special types of fat storage such as adipophilin-positive fat droplets in adipocytes (Heid et al. 1998).

Altogether, these results again emphasize the importance of standardizing the isolation of the initial cell material, culture media and methods. At the same time it is of utmost importance to develop quality control systems for MSC preparations for clinical applications and guidelines for "Good Manufacturing Practice" (GMP) have to be fulfilled. To this end, the following variables have to be taken into consideration.

16.2.1 Species

MSC have been isolated from many different species such as mouse, guinea pig, chick, rabbit, dog, pigs and human. Knowledge gained from animal models cannot always be extrapolated for human cells. It seems as if there existed many similarities with human MSC, but a systematic comparison of MSC from different species is yet elusive. Experimental data of MSC from animal models have to be validated in the human system prior to clinical application. In this chapter we will focus on human MSC.

16.2.2 Isolation of MSC from Different Sources

MSC were originally isolated from bone marrow (Friedenstein et al. 1966; Pittenger et al. 1999). In the last decade however, MSC-like cell lines could be derived from various other tissues such as umbilical cord blood (Bieback et al. 2004; Erices et al. 2000), umbilical cord matrix (Secco et al. 2008a, b; Zeddou et al. 2010), adipose tissue (Baptista et al. 2009; de Girolamo et al. 2007; Lee et al. 2004; Zuk et al. 2001), peripheral blood (Kuznetsov et al. 2001; Zvaifler et al. 2000) and skeletal muscle (Jiang et al. 2002b). Furthermore, cell preparations that fulfill the minimal criteria for MSC have also been isolated from other tissue of adult mice such as brain, liver, kidney, lung, thymus and pancreas (da Silva Meirelles et al. 2006). There is little doubt that multipotent cell populations of mesenchymal derivation reside in many tissues. Our gene expression analysis has provided clear evidence that a significant number of genes is differentially expressed in MSC isolated from specific tissue (Wagner et al. 2005a). Correspondingly, the differentiation potentials and functional implications varied widely among MSC preparations derived from different origins (Kern et al. 2006; Wagner et al. 2007a). This fact has to be taken into account when comparing results from different research groups.

16.2.3 Isolation/Depletion Using Surface Markers

Various surface markers such as STRO-1, CD73, CD105 and CD271 have been used for positive selection of MSC. Alternatively, negative selection was performed using hematopoietic surface markers such as CD34, CD45, Ter119 and glycophorin. These markers have been used alone or in combination for enrichment of fibroblast colony forming units (CFU-F). However, they do not allow direct isolation of multipotent MSC. A sophisticated comparison of the molecular features of MSC that were isolated with different enrichment methods is elusive, but it is likely that the composition of heterogenic cell preparations is significantly affected by these separation steps (Horn et al. 2008; Wagner and Ho 2007).

16.2.4 Coating of Surface and Biomaterials

Adherence to the surface of culture dishes is the most prominent feature of MSC. Properties of the surface (e.g. roughness, hydrophobicity and elasticity) significantly affect selection or differentiation potential of cell preparations (Anderson et al. 2004; Engler et al. 2006). Many protocols have applied additional protein coating of the surface (e.g. fibronectin, gelatin or collagen) to enhance cell adhesion and to mimic certain aspects of the natural extracellular microenvironment. Culture on either fibronectin or gelatin affects the morphology of the cell products after culture.

Recent studies investigate the ability of MSC to form three-dimensional mesh under specialized culture conditions. In our own hands, we observed the capacity of

bone marrow derived MSC to form round-shaped spheroid-like structures. We developed two different methods for spheroid forming: MSC were cultured as "hanging drops" with initially around 500–1,000 cells. After 24–48 h forming of spheroid-like cell aggregations could be observed. Alternatively, spheroids could be generated by culturing MSC on agarose-gel (Wuchter et al. 2009).

16.2.5 Culture Media and Serum Supplements

Culture media have a tremendous impact on gene expression and proteome of MSC (Wagner et al. 2005a, 2006). A huge arsenal of basal culture media is available and many different media have successfully been used for isolation of MSC in different laboratories. Furthermore, there is evidence that oxygen tension plays an important role and that hypoxia accelerates MSC differentiation (Ren et al. 2006).

So far most culture protocols for MSC preparation contain serum additives. Serum concentrations usually vary between 2 and 20%. Most studies have used fetal calf serum (FCS). Concerns regarding BSE, other infectious complications and host immune reactions have fueled investigation of alternative culture supplements. Recently, several groups developed alternative culture protocols for the expansion of MSC based on reagents of human origin (i.e. serum, plasma, platelet lysate etc.) that replaced fetal bovine serum (Bieback et al. 2009; Kocaoemer et al. 2007; Lange et al. 2007; Müller et al. 2006; Schallmoser et al. 2007; Stute et al. 2004). The impact of these supplements on the composition of cell preparations is yet unknown but different growth kinetics and cell morphology indicate their relevance. The development of a chemically defined and serum free growth medium would therefore substantially contribute to standardized MSC preparations.

16.2.6 In Vitro Cultivation (Passage, Density and Cryopreservation)

MSC can be passaged in vitro for a limited number of times before they become senescent and stop proliferation. As a matter of fact, molecular profiles and functional features of MSC are significantly affected by this process of cellular aging (DiGirolamo et al. 1999; Fehrer et al. 2006; Javazon et al. 2004; Wagner et al. 2008, 2009). Cell density of cultures seems to be crucial, too. Once grown to confluence, MSC have been shown to lose some of their differentiation potential (Colter et al. 2001; Sotiropoulou et al. 2006). Furthermore, MSC are often cryopreserved with DMSO in liquid nitrogen. There is evidence that cryopreserved and non-cryopreserved MSC possess the same differentiation potential, but an effect on their biological properties cannot be excluded (Kotobuki et al. 2005; Wang and Scott 1993).

16.3 Characteristics and Properties of MSC

16.3.1 Cellular Markers

MSC are often isolated from the marrow as plastic-adherent cell fraction without specific enrichment. Some groups however described markers for the isolation of MSC from primary human and murine tissues, such as STRO-1 (Simmons and Torok-Storb 1991), CD271 (low-affinity nerve growth factor receptor) (Quirici et al. 2002), CD73 (SH3, SH4) and CD105 (endoglin, SH2) (Sabatini et al. 2005), whereas CD45, Ter119 and glycophorin A (CD 235) are used for negative selections of MSC (Jiang et al. 2002a). Buhring et al. described another panel of surface markers, including platelet-derived growth factor receptor-D (CD140b), HER-2/erbB2 (CD340) and frizzled-9 (CD349), within the CD271-bright population (Buhring et al. 2007). All these markers might lead to an enrichment of MSC, but the resulting cell populations are still heterogeneous and the majority of isolated cells will not give rise to fibroblast colony-forming units (CFU-F). So far, there is no commonly accepted set of markers that distinctively describes MSC.

To address this problem the International Society for Cellular Therapy (ISCT) proposed three minimal criteria to define MSC (Dominici et al. 2006):

1. MSC must be plastic-adherent if maintained in standard culture conditions,
2. MSC must express CD73, CD90 and CD105, and lack expression of hematopoietic markers such as CD14 or CD11b, CD19 or CD79a, CD34, CD45, HLA-DR and
3. MSC must be capable of differentiation into osteoblasts, adipocytes and chondroblasts under in vitro differentiating conditions.

Neither morphologic characteristics nor specific surface markers can reliably discern the multipotent subset in MSC preparations. Using a panel of 22 surface markers including the above mentioned, there was no significant phenotypic difference between MSC and human fibroblast cell lines (HS68 and NHDF) detectable (Wagner et al. 2005a). Osteogenic, adipogenic and chondrogenic differentiation was exclusively observed in MSC preparations, but not in differentiated fibroblasts (Wagner et al. 2006). Thus, MSC-populations cannot be identified by these surface markers. Taken together, there remains an urgent need for the standardization of isolation- and culture-protocols in order to gain comparable results among different laboratories. Hence, the above mentioned minimal criteria of the ISCT are necessary and helpful, but not sufficient.

16.3.2 Gene Expression Profiling and Proteomics

Gene expression analysis has provided another dimension for the molecular characterization of cell preparations. We have compared gene expression profiles of MSC derived from bone marrow, adipose tissue and cord blood (Wagner et al. 2006, 2007a). Initial analysis demonstrated a consistent up-regulation of at least 25 well-characterized

genes in all MSC preparations, irrespective of origin and culture conditions. These genes included fibronectin 1 (FN1) and other extracellular matrix proteins (GPC4, LTBP1, ECM2, CSPG2) as well as transcription factors (nuclear factor I/B [NFIB]), homeo box genes (HOXA5 and HOXB6) and inhibitor of differentiation/DNA binding (ID1). However, none of these genes alone was specific for MSC and we have not been able to define a unique marker or marker constellation for MSC. Furthermore, we analyzed the proteome of MSC. One hundred thirty-six protein-spots were unambiguously identified by MALDI-TOF-MS. Most of the identified proteins up-regulated in MSC play a role in cytoskeleton, protein folding and metabolism. Candidate genes should be highly expressed and localized on the cell surface. In contrast, transcription factors and regulators of signal transduction are often scarcely expressed and the use of extracellular proteins is unfavourable for quality control purposes.

These results indicate that a single genomic or proteomic marker is not sufficient to define multipotent cell populations. It seems more likely, that it takes a combination of markers to reliably define MSC.

16.3.3 A Novel Type of Cell-Junctions Between MSC

We demonstrated that bone marrow derived MSC under in vitro conditions are interconnected by special villiform-to-vermiform cytoplasmatic protrusions and invaginations, termed *processus adhaerentes*, which tight-fittingly insert into deep plasma membrane invaginations, often forming batteries of interdigitating cell-cell connections with long cuff-like junctions (Wuchter et al. 2007). Cell junctions connect MSC in the intercellular space with small puncta adhaerentia. Tentacle-like cell processes could be observed that made junctional contacts with up to eight other MSC, and over distances exceeding 400 μm. Alternatively, they can also form deep plasma membrane invaginations in neighbouring cells (*recessus adhaerentes*) (Fig. 16.2). This novel type of cell junctions is characterized by a molecular complement comprising N-cadherin and cadherin-11, in combination with the cytoplasmic plaque proteins α- and β-catenin, together with p120ctn and plakoglobin. The long *processus adhaerentes* interconnect several distant MSC to formations of a closer packed cell assembly. The frequency and morphology of these junctional complexes are greatly affected by culture conditions (unpublished observation). A similar type of homotypic cell–cell interaction has previously been described by Werner W. Franke and co-workers in studies of primary mesenchymal cells of the mouse embryo (Franke et al. 1983). These findings indicate that this novel type of cell junctions is more wide spread in embryonal and other tissues and might be relevant for the primitive function of MSC and heterotypic interaction with other cell types.

16.3.4 Co-culture of MSC and Hematopoietic Stem Cells

The interaction between human hematopoietic stem cells (HSC) and their niche plays a key role in regulating maintenance of “stemness” and differentiation. MSC

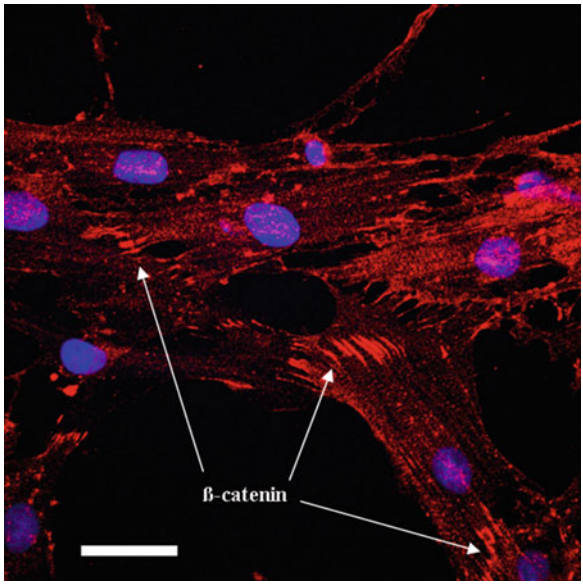


Fig. 16.2 MSC are interconnected by junctional complexes. Immunofluorescent staining of ß-catenin (*red*), nuclei are stained in *blue* (scale bar 100 μm).

feeder-layer can serve as surrogate model for the hematopoietic stem cell (HSC) niche in vitro (Wagner et al. 2005b, 2007a, c; Walenda et al. 2010). We further analyzed the intercellular junctional complexes between HSC from umbilical cord blood and MSC. Using confocal laser scanning in combination with deconvolution and volume rendering software, we were able to produce 3D-images of intercellular junctions between HSC and MSC. We used a panel of antibodies specific for various components of tight, gap and adherens junctions and could show that intercellular connections between HSC and MSC are mainly realized by podia formation of the HSC linking to the adjacent MSC. These podia vary greatly in length and shape (uropodia, filopodia). Along these podia and especially at the contact zone to the MSC, we have identified the cytoplasmic plaque proteins alpha- and beta-catenin and protein $p120^{ctn}$, as well as the transmembrane glycoprotein N-cadherin (Wuchter et al. 2008). Cell division kinetic of HSC was increased when cocultured with MSC and the rate of CD34+ cells remained higher compared to monoculturing of HSC in the same culture-medium (Wagner et al. 2007b). These results underline that direct cellular contact is essential for homing and adhesion of HSC to the cellular niche and subsequently for the regulation of self-renewal versus differentiation in HSC.

16.4 Differentiation Capacity

“Pluripotent” stem cells give rise to diverse cell types of all three germ layers. In contrast, “multipotent” stem cells can only produce related cell types of the same germinal layer.

A subtype of bone marrow derived cells, called "multipotent adult progenitor cells" (MAPC) has been suggested by the group of Verfaillie to be able to generate cells with characteristics of visceral mesoderm, neuro-ectoderm and endoderm (Jiang et al. 2002b, 2003; Zeng et al. 2006). However, the validity and reproducibility of these data has been discussed controversely (Check 2007). It cannot be excluded, that the pluripotency in these cell preparations resembles some kind of "culture artefact" that occurs under long-term culture expansion.

Kogler et al. described another subset of MSC derived from human cord blood that they called "unrestricted somatic stem cells" (USSC) (Kogler et al. 2004). These cells were able to differentiate into many cell types, even hepatocytes and cardiomyocytes. These experiments suggest that culture conditions and specific modifications of the isolation protocols have a tremendous impact on the developmental potential of the populations generated, albeit the starting cell populations could be phenotypically identical. Ratajczak and coworkers recently identified a population of CXCR4(+) "very small embryonic like stem cells" (VSEL) in murine bone marrow and human cord blood (Halasa et al. 2008; Kucia et al. 2006a). They hypothesized that these cells are deposited during development in BM as a mobile pool of circulating pluripotent stem cells that play a pivotal role in postnatal tissue turnover, both of non-hematopoietic and hematopoietic tissues (Kucia et al. 2008a). These cells could be mobilized from BM and circulate in peripheral blood during tissue/organ injury in an attempt to regenerate damaged organs (Kucia et al. 2008b). However, if these cells are mobilized at the wrong time and migrate to the wrong place they may contribute to the development of several pathologies, including tumor formation (Kucia et al. 2006b).

On the other hand, the validity of all the initial experiments on transdifferentiation potentials of other adult stem cells, for example hematopoietic stem cells (HSC), has been severely challenged in the last few years. Some of the experiments could not be reproduced by other groups (Check 2007; Morshead et al. 2002; Raedt et al. 2007; Ying et al. 2002). In other cases, the assumed process of transdifferentiation under closer examination finally turned out to be a product of spontaneous cell fusion (Terada et al. 2002).

Taken together, it is commonly accepted, that MSC are multipotent with differentiation potential towards bone, cartilage and adipose lineage. Nevertheless, this does not rule out the possibility that scarce mesenchymal progenitor cell populations in bone marrow may exist, that truly possess further differentiation potential.

16.5 Replicative Senescence and Aging of MSC

Culture expansion of MSC is limited as well as for any other normal, somatic cell. After a certain number of cell divisions they enter a senescent state and ultimately stop proliferation. These cells are not dead and can be maintained in this non-proliferative state for months. This phenomenon was first described in the 1960s by Leonard Hayflick (1965). Cellular senescence is accompanied by morphologic

changes: cell enlargement and a flat "fried egg morphology". Notably, the in vitro differentiation potential also decays after long-term culture expansion. Furthermore, replicative senescence of MSC is accompanied by various gene expression changes that are even consitent under different culture conditions (Schallmoser et al. 2009; Wagner et al. 2008)

Various molecular pathways have been implicated in senescence such as DNA damage, accumulation of the cyclin-dependent kinase inhibitor p16INK4a and oxidative stress (Ho et al. 2005; Janzen et al. 2006; Kiyono et al. 1998). Progressive shortening of the telomeres or modified telomeric structures have been proposed to be the main trigger for replicative senescence - with every cell division the number of telomere repeats decreases and this has been suggested as a kind of internal clock (Bonab et al. 2006; Fehrer and Lepperdinger 2005; Lansdorp 2008). It is however still controversially discussed if telomere shortening is the only initiating mechanism for replicative senescence or if it rather resembles an effect of senescence (Di Donna et al. 2003; Kiyono et al. 1998; Masutomi et al. 2003; Zimmermann et al. 2004). Alternatively, it has been proposed that molecular switches such as epigenetic modifications might play a central role for regulation of cellular aging (Bork et al. 2010; Chambers et al. 2007; Nilsson et al. 2005; Shibata et al. 2007; Suzuki et al. 2008; Wilson and Jones 1983; Young and Smith 2001).

Since the first discovery of the Hayflick limit it has been speculated if replicative senescence is involved in aging of the whole organism. Indeed, several authors have shown an inverse relationship between donor age and the replicative life span *in vitro* for fibroblasts as well as for MSC (Mareschi et al. 2006; Schneider and Mitsui 1976; Stenderup et al. 2003). These studies are hampered by large inter-individual differences and MSC content and therefore necessitate high numbers of donor samples (Bonab et al. 2006; Cristofalo et al. 1998; Wagner et al. 2009). Overall, MSC from elderly people seem to have a slower proliferation rate already at the initial cell passage and that they contain larger and flatter cells in comparison to cells from younger donors (Roobrouck et al. 2008). Zhou and co-workers demonstrated that the number of cells that staining positive for senescence-associated beta-galactosidase is significantly higher in samples from elderly donors in comparison to younger donors (Zhou et al. 2008). Another observation, which supports age-related effects on replicative senescence is that the frequency of cells with colony forming potential declines at higher ages (Baxter et al. 2004; Stolzing et al. 2008). The two processes are also related on a molecular basis: genes which are up-regulated in long-term culture are also up-regulated in elderly people (Wagner et al. 2008, 2009).

We have recently analyzed DNA methylation profiles of MSC using the HumanMethylation27 BeadChip (Bork et al. 2010). This platform represents 27,578 CpG sites that are associated with promoter regions of more than 13,500 annotated genes. Our results revealed that overall the methylation remained rather constant throughout long-term culture for 2–3 month. However, specific CpG islands were either hyper-methylated or hypo-methylated and the same changes were also verified in independent donor samples. Differentially methylated regions correlated with various developmental genes and there was an association with differential methylation between samples from young and elderly donors. These results support the

notion, that replicative senescence and aging represent developmental processes that are regulated by similar epigenetic modifications.

Despite such molecular insights it is still only scarcely understood how long-term culture affects the composition of MSC preparations and five processes seem to be involved (Wagner et al. 2010a, b): (1) MSC are composed of sub-populations with different proliferation rates and therefore the heterogeneity notoriously changes in the course of *in vitro* culture; (2) cells in culture acquire mutations and other stochastic cellular defects; (3) self-renewal of MSC may be impaired in the artificial environment of a culture dish leading to gradual differentiation; (4) the number of cell divisions might be restricted – for example by telomere loss under culture conditions or (5) replicative senescence might be associated with the aging process of the organism as mentioned above.

Due to the functional implications of long-term culture there is a growing perception that this process has to be taken into account – especially for clinical applications. On the other hand the state of replicative senescence is poorly defined by the number of population doublings or even by the number of passages. Reliable markers for cellular aging are urgently needed.

16.6 Potential Applications for Therapies

Theoretically, MSC can be isolated from a small aspirate of BM or tissue samples and expanded in vitro. Preliminary studies suggest that MSC preferentially home to damaged tissue and therefore have therapeutic potential (LeBlanc 2006). The website www.ClinicalTrials.gov of the National Institutes of Health (Bethesda, MD, USA) is currently listing more than 100 active clinical trials in which MSC are involved. Clinical applications include treatment of such different entities as steroid refractory graft versus host disease (GvHD), osteonecrosis, articular cartilage defects, severe chronic myocardial ischemia, decompensated liver cirrhosis, multiple sclerosis, Type I and II diabetes mellitus, Lupus nephritis, Crohn's disease and more. So far the studies revealed no serious side effects upon transplantation of MSC. However, it is unclear if the beneficial effects that have been observed in some studies are really due to true transdifferentiation of MSC into the damaged tissue cells. For example, clinical trials using MSC for myocardial infarction have proceeded rapidly, but there is little or no evidence for the differentiation of MSC to coupled cardiomyocytes (Caplan and Dennis 2006; Grinnemo et al. 2006; Stamm et al. 2006). The beneficial effects shown in some of these studies might be attributable to paracrine anti-inflammatory signalling or stimulation of endogenous repair processes by the injected cells (Mazhari and Hare 2007). The precise underlying mechanisms are yet unknown. This lack of knowledge might not prevent application of MSC in clinical settings if there are benefits for the patient and if there are no or minimal side effects.

At present, the most promising clinical studies make use of the immunomodulatory effects of MSC. In vitro data suggest that MSCs have low inherent

immunogenicity as they induce little, if any, proliferation of allogeneic lymphocytes. Instead, MSCs appear to be immunosuppressive in vitro (Le Blanc and Ringdén 2007). A recent multicenter, phase II experimental study enrolled 55 patients with steroid-resistant, severe, acute GvHD. Patients were treated with mesenchymal stem cells, derived with the European Group for Blood and Marrow Transplantation ex-vivo expansion procedure. Thirty patients had a complete response and nine showed improvement. Complete responders had lower transplantation-related mortality 1 year after infusion than did patients with partial or no response (37% vs. 72%; $p=0.002$) and higher overall survival 2 years after haematopoietic stem-cell transplantation (53% vs. 16%; $p=0.018$) (Le Blanc et al. 2008). In a pilote study LeBlanc et al. could furthermore demonstrate, that transplantation of mesenchymal stem cells enhanced the engraftment of hematopoietic stem cells: seven patients underwent treatment with mesenchymal stem cells together with allogeneic hematopoietic stem cell transplantation, resulting in fast engraftment of absolute neutrophil count and platelets and 100% donor chimerism, even in three patients regrafted for graft failure/rejection (Le Blanc et al. 2007).

However, recent data indicated that MSC may undergo spontaneous transformation following long-term culture (Meza-Zepeda et al. 2008; Rosland et al. 2009; Rubio et al. 2005). Recent reports indicate that sarcoma can evolve from murine MSC cultures (Li et al. 2010; Tolar et al. 2007). Karnoub and coworkers demonstrated that bone-marrow-derived human mesenchymal stem cells, when mixed with otherwise weakly metastatic human breast carcinoma cells, cause the cancer cells to increase their metastatic potency greatly when this cell mixture is introduced into a subcutaneous site and allowed to form a tumour xenograft (Karnoub et al. 2007). Therefore, malignant transformation of therapeutic cell preparations is like the “Sword of Damocles” and may jeopardize the use of MSC as therapeutic tools. Further studies need to address and clarify this issue, before MSC can be used as a standard-option for clinical use.

16.7 Conclusions and Future Development in Research

The lack of common standards and universal guidelines for the preparation of MSC has greatly hampered further progress. The quality of preparations from different laboratories varies significantly and the cell products are notoriously heterogeneous regarding the source and freshness of starting material, isolation protocols, culture-conditions, number of passages upon culture, etc. There is an urgent need for the development of molecular markers and universal criteria for quality control of the starting cell populations as well as for the cell products after expansion. Such a comprehensive approach might also be helpful to clear the role of the above mentioned subpopulations, i.e. MAPC, USSC and VSEL. For clinical use, a clear definition of the transplanted cell populations in conjunction with serum-free culture media and close quality controls throughout the whole production-process is essential.

Even though there is so far no convincing evidence that MSC themselves are involved in the process of tissue repair by transdifferentiation, they probably contribute to this process by providing a supportive microenvironment for other cell types that are directly involved, including other types of adult stem cells. At present, three major fields can be identified, in which MSC can and will be used for therapeutic purposes: (I) tissue repair (Le Blanc 2006; Mazhari and Hare 2007; Müller et al. 2008b); (II) therapy of chronic graft vs. host disease (GvHD) (Aksu et al. 2008; Le Blanc et al. 2008; Tian et al. 2008), and (III) enhancement of hematopoietic stem cell engraftment in an allogeneic transplant setting (Le Blanc et al. 2007; Müller et al. 2008a). The underlying key mechanism in all the three fields is not completely understood, but probably a result of MSC-induced immunosuppression. We therefore need aggressive attempts to better understand the immunomodulatory mechanisms of MSC.

Acknowledgements This work was supported by the German Ministry of Education and Research (BMBF) within the supporting program "cell based regenerative medicine" (START-MSC2 and CB-HERMES), the German Research Foundation DFG (SFB 873), the Network for Aging Research (NAR, Heidelberg), the Heidelberg Academy of Sciences (WIN-Kolleg) and the Stem Cell Network North Rhine Westphalia. We would like to thank Dr. Rainer Saffrich for excellent image acquisition.

References

Aksu AE, Horibe E, Sacks J, Ikeguchi R, Breitinger J, Scozio M, Unadkat J, Feili-Hariri M (2008) Co-infusion of donor bone marrow with host mesenchymal stem cells treats GvHD and promotes vascularized skin allograft survival in rats. Clin Immunol 127(3):348–358

Anderson DG, Levenberg S, Langer R (2004) Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. Nat Biotechnol 22:863–866

Baptista LS, do Amaral RJ, Carias RB, Aniceto M, Claudio-da-Silva C, Borojevic R (2009) An alternative method for the isolation of mesenchymal stromal cells derived from lipoaspirate samples. Cytotherapy 11(6):706–715

Baxter MA, Wynn RF, Jowitt SN, Wraith JE, Fairbairn LJ, Bellantuono I (2004) Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. Stem Cells 22:675–682

Bieback K, Kern S, Kluter H et al (2004) Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 22:625–634

Bieback K, Hecker A, Kocaömer A, Lannert H, Schallmoser K, Strunk D, Klüter H (2009) Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. Stem Cells 27(9):2331–2341

Bieback K, Wuchter P, Besser D, Franke W, Becker M, Ott M, Pacher M, Ma N, Stamm C, Klüter H, Müller A, Ho AD; START-MSC Consortium (2012) Mesenchymal stromal cells (MSCs): science and f(r)iction. J Mol Med 90(7):773–782

Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B (2006) Aging of mesenchymal stem cell in vitro. BMC Cell Biol 7:14

Bork S, Pfister S, Witt H, Horn P, Korn B, Ho AD, Wagner W (2010) DNA methylation pattern changes upon long-term culture and aging of human mesenchymal stromal cells. Aging Cell 9(1):54–63

Buhring HJ, Battula VL, Treml S et al (2007) Novel markers for the prospective isolation of human MSC. Ann N Y Acad Sci 1106:262–271

Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641–650
Caplan AI, Dennis JE (2006) Mesenchymal stem cells as trophic mediators. J Cell Biochem 98:1076–1084
Chambers SM, Shaw CA, Gatza C, Fisk CJ, Donehower LA, Goodell MA (2007) Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. PLoS Biol 5:201
Check E (2007) The hard copy. News feature. Nature 446:485–486
Colter DC, Sekiya I, Prockop DJ (2001) Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci USA 98:7841–7845
Cristofalo VJ, Allen RG, Pignolo RJ, Martin BG, Beck JC (1998) Relationship between donor age and the replicative lifespan of human cells in culture: a reevaluation. Proc Natl Acad Sci USA 95:10614–10619
da Silva ML, Chagastelles PC, Nardi NB (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 119:2204–2213
de Girolamo L, Sartori MF, Albisetti W, Brini AT (2007) Osteogenic differentiation of human adipose-derived stem cells: comparison of two different inductive media. J Tissue Eng Regen Med 1(2):154–157
Di Donna S, Mamchaoui K, Cooper RN, Seigneurin-Venin S, Tremblay J, Butler-Browne GS, Mouly V (2003) Telomerase can extend the proliferative capacity of human myoblasts, but does not lead to their immortalization. Mol Cancer Res 1:643–653
DiGirolamo CM, Stokes D, Colter D et al (1999) Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 107:275–281
Dominici M, Le Blanc K, Mueller I et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317
Engler AJ, Sen S, Sweeney HL et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126:677–689
Erices A, Conget P, Minguell JJ (2000) Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol 109:235–242
Fehrer C, Lepperdinger G (2005) Mesenchymal stem cell aging. Exp Gerontol 40:926–930
Fehrer C, Laschober G, Lepperdinger G (2006) Aging of murine mesenchymal stem cells. Ann N Y Acad Sci 1067:235–242
Franke WW, Grund C, Jackson BW et al (1983) Formation of cytoskeletal elements during mouse embryogenesis. IV. Ultrastructure of primary mesenchymal cells and their cell-cell interactions. Differentiation 25:121–141
Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV (1966) Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 16:381–390
Friedenstein AJ, Petrakova KV, Kurolesova AI et al (1968) Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation 6:230–247
Friedenstein AJ, Chailakhyan RK, Latsinik NV et al (1974) Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 17:331–340
Grinnemo KH, Månsson-Broberg A, Leblanc K, Corbascio M, Wärdell E, Siddiqui AJ, Hao X, Sylvén C, Dellgren G (2006) Human mesenchymal stem cells do not differentiate into cardiomyocytes in a cardiac ischemic xenomodel. Ann Med 38:144–153
Halasa M, Baskiewicz-Masiuk M, Dabkowska E et al (2008) An efficient two-step method to purify very small embryonic-like (VSEL) stem cells from umbilical cord blood (UCB). Folia Histochem Cytobiol 46(2):239–243
Hayflick L (1965) The limited in vitro lifetime of human diploid cell strains. Exp Cell Res 37:614–636
Heid HW, Moll R, Schwetlick I, Rackwitz HR, Keenan TW (1998) Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. Cell Tissue Res 294(2):309–321

Ho AD, Wagner W, Mahlknecht U (2005) Stem cells and ageing. The potential of stem cells to overcome age-related deteriorations of the body in regenerative medicine. EMBO Rep 6:35–38
Ho AD, Wagner W, Franke WW (2008) Heterogeneity of mesenchymal stromal cell preparations. Cytotherapy 10:320–330
Horn P, Bork S, Diehlmann A, Walenda T, Eckstein V, Ho AD, Wagner W (2008) Isolation of human mesenchymal stromal cells is more efficient by red blood cell lysis. Cytotherapy 10(7):676–685
Horwitz EM, Keating A (2000) Nonhematopoietic mesenchymal stem cells: what are they? Cytotherapy 2:387–388
Horwitz EM, Le BK, Dominici M et al (2005) Clarification of the nomenclature for MSC: the International Society for Cellular Therapy position statement. Cytotherapy 7:393–395
Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, Cheng T, DePinho RA, Sharpless NE, Scadden DT (2006) Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 443:421–426
Javazon EH, Beggs KJ, Flake AW (2004) Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 32:414–425
Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002a) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49. Corrigendum (2007) Nature 447:880–881
Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM (2002b) Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol 30:896–904
Jiang Y, Henderson D, Blackstad M, Chen A, Miller RF, Verfaillie CM (2003) Neuroectodermal differentiation from mouse multipotent adult progenitor cells. Proc Natl Acad Sci USA 100(Suppl 1):11854–11860
Johnstone B, Hering TM, Caplan AI et al (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238:265–272
Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA (2007) Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 449(7162):557–563
Kern S, Eichler H, Stoeve J et al (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24:1294–1301
Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 396:84–88
Kocaoemer A, Kern S, Kluter H et al (2007) Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. Stem Cells 25:1270–1278
Kogler G, Sensken S, Airey JA et al (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 200:123–135
Kotobuki N, Hirose M, Machida H et al (2005) Viability and osteogenic potential of cryopreserved human bone marrow-derived mesenchymal cells. Tissue Eng 11:663–673
Kucia M, Reca R, Campbell FR et al (2006a) A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 20(5):857–869
Kucia M, Zuba-Surma E, Wysoczynski M et al (2006b) Physiological and pathological consequences of identification of very small embryonic like (VSEL) stem cells in adult bone marrow. J Physiol Pharmacol 57(Suppl 5):5–18
Kucia M, Wysoczynski M, Ratajczak J et al (2008a) Identification of very small embryonic like (VSEL) stem cells in bone marrow. Cell Tissue Res 331(1):125–134
Kucia M, Wysoczynski M, Wu W et al (2008b) Evidence that very small embryonic-like stem cells are mobilized into peripheral blood. Stem Cells 26(8):2083–2092

Kuznetsov SA, Mankani MH, Gronthos S et al (2001) Circulating skeletal stem cells. J Cell Biol 153:1133–1140

Lange C, Cakiroglu F, Spiess AN et al (2007) Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine. J Cell Physiol 213(1):18–26

Lansdorp PM (2008) Telomeres, stem cells, and hematology. Blood 111:1759–1766

Le Blanc K (2006) Mesenchymal stromal cells: tissue repair and immune modulation. Cytotherapy 8(6):559–561

Le Blanc K, Ringdén O (2007) Immunomodulation by mesenchymal stem cells and clinical experience. J Intern Med 262(5):509–525

Le Blanc K, Samuelsson H, Gustafsson B, Remberger M, Sundberg B, Arvidson J, Ljungman P, Lönnies H, Nava S, Ringdén O (2007) Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. Leukemia 21(8):1733–1738

Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringdén O (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 371(9624):1579–1586

Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH (2004) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 103:1669–1675

Li Q, Hisha H, Takaki T, Adachi Y, Li M, Song C, Feng W, Okazaki S, Mizokami T, Kato J, Inaba M, Hosaka N, Maki M, Ikehara S (2010) Transformation potential of bone marrow stromal cells into undifferentiated high-grade pleomorphic sarcoma. J Cancer Res Clin Oncol 136(6):829–838

Mareschi K, Ferrero I, Rustichelli D, Aschero S, Gammaitoni L, Aglietta M, Madon E, Fagioli F (2006) Expansion of mesenchymal stem cells isolated from pediatric and adult donor bone marrow. J Cell Biochem 97:744–754

Masutomi K, Yu EY, Khurts S, Ben-Porath I, Currier JL, Metz GB, Brooks MW, Kaneko S, Murakami S, DeCaprio JA, Weinberg RA, Stewart SA, Hahn WC (2003) Telomerase maintains telomere structure in normal human cells. Cell 114:241–253

Mazhari R, Hare JM (2007) Mechanisms of action of mesenchymal stem cells in cardiac repair: potential influences on the cardiac stem cell niche. Nat Clin Pract Cardiovasc Med 4(Suppl 1):S21–S26

Meza-Zepeda LA, Noer A, Dahl JA, Micci F, Myklebost O, Collas P (2008) High-resolution analysis of genetic stability of human adipose tissue stem cells cultured to senescence. J Cell Mol Med 12:553–563

Morshead CM, Benveniste P, Iscove NN et al (2002) Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations. Nat Med 8:268–273

Müller I, Kordowich S, Holzwarth C, Spano C, Isensee G, Staiber A, Viebahn S, Gieseke F, Langer H, Gawaz MP, Horwitz EM, Conte P, Handgretinger R, Dominici M (2006) Animal serum-free culture conditions for isolation and expansion of multipotent mesenchymal stromal cells from human BM. Cytotherapy 8:437–444

Müller I, Kordowich S, Holzwarth C, Isensee G, Lang P, Neunhoeffer F, Dominici M, Greil J, Handgretinger R (2008a) Application of multipotent mesenchymal stromal cells in pediatric patients following allogeneic stem cell transplantation. Blood Cells Mol Dis 40(1):25–32

Müller I, Vaegler M, Holzwarth C, Tzaribatchev N, Pfister SM, Schütt B, Reize P, Greil J, Handgretinger R, Rudert M (2008b) Secretion of angiogenic proteins by human multipotent mesenchymal stromal cells and their clinical potential in the treatment of avascular osteonecrosis. Leukemia 22(11):2054–2061

Nakahara H, Bruder SP, Haynesworth SE et al (1990) Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum. Bone 11:181–188

Nilsson O, Mitchum RD Jr, Schrier L, Ferns SP, Barnes KM, Troendle JF, Baron J (2005) Growth plate senescence is associated with loss of DNA methylation. J Endocrinol 186:241–249

Pittenger MF, Mackay AM, Beck SC et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147
Quirici N, Soligo D, Bossolasco P et al (2002) Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp Hematol 30:783–791
Raedt R, Pinxteren J, Van Dycke A et al (2007) Differentiation assays of bone marrow-derived multipotent adult progenitor cell (MAPC)-like cells towards neural cells cannot depend on morphology and a limited set of neural markers. Exp Neurol 203:542–554
Ren H, Cao Y, Zhao Q et al (2006) Proliferation and differentiation of bone marrow stromal cells under hypoxic conditions. Biochem Biophys Res Commun 347:12–21
Roobrouck VD, Ulloa-Montoya F, Verfaillie CM (2008) Self-renewal and differentiation capacity of young and aged stem cells. Exp Cell Res 314:1937–1944
Rosland GV, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, Mysliwietz J, Tonn JC, Goldbrunner R, Lonning PE, Bjerkvig R, Schichor C (2009) Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. Cancer Res 69:5331–5339
Rubio D, Garcia-Castro J, Martin MC de la FR, Cigudosa JC, Lloyd AC, Bernad A (2005) Spontaneous human adult stem cell transformation. Cancer Res 65:3035–3039
Sabatini F, Petecchia L, Tavian M, Jodon de Villeroché V, Rossi GA, Brouty-Boyé D (2005) Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities. Lab Invest 85:962–971
Schallmoser K, Bartmann C, Rohde E, Reinisch A, Kashofer K, Stadelmeyer E, Drexler C, Lanzer G, Linkesch W, Strunk D (2007) Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. Transfusion 47(8):1436–1446
Schallmoser K, Bartmann C, Rodhe E, Bork S, Guelly C, Obenauf AC, Reinisch A, Horn P, Ho AD, Strunk D, Wagner W (2009) Replicative senescence-associated gene expression changes in mesenchymal stromal cells are similar under different culture conditions. Haematologica 95(6):867–874
Schneider EL, Mitsui Y (1976) The relationship between in vitro cellular aging and in vivo human age. Proc Natl Acad Sci 73:3584–3588
Secco M, Zucconi E, Vieira NM, Fogaça LL, Cerqueira A, Carvalho MD, Jazedje T, Okamoto OK, Muotri AR, Zatz M (2008a) Mesenchymal stem cells from umbilical cord: do not discard the cord! Neuromuscul Disord 18(1):17–18
Secco M, Zucconi E, Vieira NM, Fogaça LL, Cerqueira A, Carvalho MD, Jazedje T, Okamoto OK, Muotri AR, Zatz M (2008b) Multipotent stem cells from umbilical cord: cord is richer than blood! Stem Cells 26(1):146–150
Shibata KR, Aoyama T, Shima Y, Fukiage K, Otsuka S, Furu M, Kohno Y, Ito K, Fujibayashi S, Neo M, Nakayama T, Nakamura T, Toguchida J (2007) Expression of the p16INK4A gene is associated closely with senescence of human mesenchymal stem cells and is potentially silenced by DNA methylation during in vitro expansion. Stem Cells 25:2371–2382
Simmons PJ, Torok-Storb B (1991) Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood 78:55–62
Sotiropoulou PA, Perez SA, Salagianni M et al (2006) Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells 24:462
Stamm C, Liebold A, Steinhoff G et al (2006) Stem cell therapy for ischemic heart disease: beginning or end of the road? Cell Transplant 15(Suppl 1):S47–S56
Stenderup K, Justesen J, Clausen C, Kassem M (2003) Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone 33:919–926
Stolzing A, Jones E, McGonagle D, Scutt A (2008) Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. Mech Ageing Dev 129:163–173
Stute N, Holtz K, Bubenheim M et al (2004) Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use. Exp Hematol 32:1212–1225

Suzuki T, Farrar JE, Yegnasubramanian S, Zahed M, Suzuki N, Arceci RJ (2008) Stable knockdown of PASG enhances DNA demethylation but does not accelerate cellular senescence in TIG-7 human fibroblasts. Epigenetics 3:281–291

Terada N, Hamazaki T, Oka M et al (2002) Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542–545

Tian Y, Deng YB, Huang YJ, Wang Y (2008) Bone marrow-derived mesenchymal stem cells decrease acute graft-versus-host disease after allogeneic hematopoietic stem cells transplantation. Immunol Invest 37(1):29–42

Tolar J, Nauta AJ, Osborn MJ, Panoskaltsis Mortari A, McElmurry RT, Bell S, Xia L, Zhou N, Riddle M, Schroeder TM, Westendorf JJ, McIvor RS, Hogendoorn PC, Szuhai K, Oseth L, Hirsch B, Yant SR, Kay MA, Peister A, Prockop DJ, Fibbe WE, Blazar BR (2007) Sarcoma derived from cultured mesenchymal stem cells. Stem Cells 25(2):371–379

Wagner W, Ho AD (2007) Mesenchymal stem cell preparations – comparing apples and oranges. Stem Cell Rev 3:239–248

Wagner W, Wein F, Seckinger A et al (2005a) Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol 33:1402–1416

Wagner W, Saffrich R, Wirkner U et al (2005b) Hematopoietic progenitor cells and cellular microenvironment: behavioral and molecular changes upon interaction. Stem Cells 23:1180–1191

Wagner W, Feldmann RE Jr, Seckinger A, Maurer MH, Wein F, Blake J, Krause U, Kalenka A, Burgers HF, Saffrich R, Wuchter P, Kuschinsky W, Ho AD (2006) The heterogeneity of human mesenchymal stem cell preparations – evidence from simultaneous analysis of proteomes and transcriptomes. Exp Hematol 34:536–548

Wagner W, Roderburg C, Wein F et al (2007a) Molecular and secretory profiles of human mesenchymal stromal cells and their abilities to maintain primitive hematopoietic progenitors. Stem Cells 10:2638–2647

Wagner W, Wein F, Roderburg C et al (2007b) Adhesion of hematopoietic progenitor cells to human mesenchymal stem cells as a model for cell-cell interaction. Exp Hematol 35:314–325

Wagner W, Wein F, Roderburg C et al (2007c) Adhesion of human hematopoietic progenitor cells to mesenchymal stromal cells involves CD44. Cells Tissues Organs 188(1–2):160–169

Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, Ho AD (2008) Replicative senescence of mesenchymal stem cells – a continuous and organized process. PLoS One 5:e2213

Wagner W, Bork S, Horn P, Krunic D, Walenda T, Diehlmann A, Benes V, Blake J, Huber FX, Eckstein V, Boukamp P, Ho AD (2009) Aging and replicative senescence have related effects on human stem and progenitor cells. PLoS One 4:e5846

Wagner W, Bork S, Lepperdinger G, Joussen S, Ma N, Strunk D, Koch C (2010a) How to track cellular aging of mesenchymal stromal cells. Aging 2:224–230

Wagner W, Ho AD, Zenke M (2010b) Different facets of aging in human mesenchymal stem cells. Tissue Eng Part B Rev 16:445–453

Walenda T, Bork S, Horn P, Wein F, Saffrich R, Diehlmann A, Eckstein V, Ho AD, Wagner W (2010) Co-culture with mesenchymal stromal cells increases proliferation and maintenance of hematopoietic progenitor cells. J Cell Mol Med 14(1–2):337–350

Wang H, Scott RE (1993) Inhibition of distinct steps in the adipocyte differentiation pathway in 3T3 T mesenchymal stem cells by dimethyl sulphoxide (DMSO). Cell Prolif 26:55–66

Wilson VL, Jones PA (1983) DNA methylation decreases in aging but not in immortal cells. Science 220:1055–1057

Wuchter P, Boda-Heggemann J, Straub BK, Grund C, Kuhn C, Krause U, Seckinger A, Peitsch WK, Spring H, Ho AD, Franke WW (2007) Processus and recessus adhaerentes: giant adherens cell junction systems connect and attract human mesenchymal stem cells. Cell Tissue Res 328:499–514

Wuchter P, Saffrich R, Wagner W, Wein F, Schubert M, Eckstein V, Ho AD (2008) Human hematopoietic stem cells and leukemic cells form cadherin-catenin based junctional complexes with mesenchymal stromal cells. Blood (ASH Annu Meet Abstr) 112:1367

Wuchter P, Saffrich R, Ludwig A, Schubert M, Eckstein V, Ho AD (2009) Cellular interaction between human mesenchymal stem cells and hematopoietic stem cells in 2D- and 3D-culture-systems. Blood (ASH Annu Meet Abstr) 114:1442

Ying QL, Nichols J, Evans EP et al (2002) Changing potency by spontaneous fusion. Nature 416:545–548

Young JI, Smith JR (2001) DNA methyltransferase inhibition in normal human fibroblasts induces a p21-dependent cell cycle withdrawal. J Biol Chem 276:19610–19616

Zeddou M, Briquet A, Relic B, Josse C, Malaise MG, Gothot A, Lechanteur C, Beguin Y (2010) The umbilical cord matrix is a better source of mesenchymal stem cells (MSC) than the umbilical cord blood. Cell Biol Int 34(7):693–701

Zeng L, Rahrmann E, Hu Q et al (2006) Multipotent adult progenitor cells from swine bone marrow. Stem Cells 24:2355–2366

Zhou S, Greenberger JS, Epperly MW, Goff JP, Adler C, Leboff MS, Glowacki J (2008) Age-related intrinsic changes in human bone-marrow-derived mesenchymal stem cells and their differentiation to osteoblasts. Aging Cell 7:335–343

Zimmermann S, Glaser S, Ketteler R, Waller CF, Klingmuller U, Martens UM (2004) Effects of telomerase modulation in human hematopoietic progenitor cells. Stem Cells 22:741–749

Zuk PA, Zhu M, Mizuno H et al (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228

Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, Maini RN (2000) Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res 2(6):477–488

Chapter 17
Musculoskeletal Stem Cells

Gerben M. van Buul and Gerjo J.V.M. van Osch

Abstract Probably the most striking example of musculoskeletal regeneration is the growing of an entire limb by a salamander, after it is traumatically amputated. This classic example unfortunately doesn't apply to humans, but (local) stem cells are indispensable in providing a renewable cell source for physiological tissue homeostasis and regeneration after musculoskeletal tissue injury. Stem cells have been isolated from the following musculoskeletal tissues: bone marrow, adipose tissue, periosteum, perichondrium, tendons, ligaments, muscle, cartilage, bone and synovial membrane or -fluid. We chose to refer to these cells as mesenchymal progenitor cells (MPCs). Cells from these different tissues are generally isolated by mincing the tissue followed by enzymatic digestion. Overall, the cells are positive for CD44, CD90, CD105, (CD146), CD166 and STRO-1 and negative for CD31, CD34, CD45 and CD117. On the whole, cell yields from these tissues and proliferation capacities of these cells appear to be within the same order of magnitude. Cells derived from the various musculoskeletal tissues have all been shown to have a multi-lineage differentiation potential, although they do show differentiation preferences, in general for differentiating towards the tissue they were originally derived from. Regenerative capacities of local stem cells are based on two characteristics. In the first place, they have the ability to differentiate into mature tissue cells, thereby contributing to new tissue formation. As a second quality, local stem cells secrete trophic factors that may be responsible for another mechanism of stem cell-mediated tissue repair. These trophic factors are capable of attracting (more) stem cells to the damaged area and they can play an immunomodulatory role. Musculoskeletal stem cells posses a huge capacity for application in regenerative medicine.

G.M. van Buul, M.D. • G.J.V.M. van Osch, Ph.D. (✉)
Departments of Orthopaedics and Radiology, Erasmus MC,
University Medical Center Rotterdam, Molewaterplein 50,
3015 GE, Rotterdam, The Netherlands
e-mail: g.vanosch@erasmusmc.nl

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_17,

17.1 Introduction

Regeneration of tissues happens on a daily basis throughout our lives. For many years scientist have been trying to elucidate this process, which is delicately regulated by molecular and cellular events. The growing of an entire limb by a salamander after its traumatic amputation is one of the most remarkable examples of musculoskeletal regeneration. This process is called epimorphosis, and is characterised by cellular dedifferentiation and proliferation at the wound site (Brockes and Kumar 2002); Local mesenchymal cells lose their phenotype, start proliferating as blastemal cells followed by redifferentiation in order to form the tissues required for the newly formed limb (Brockes and Kumar 2002). This example, which is very appealing to one's imagination, regrettably doesn't relate to humans. Still, (local) stem cells are indispensable in providing a renewable cell source for physiological tissue homeostasis and regeneration after tissue injury (Lin et al. 2008). In the 1960s, the work of Alexander Friedenstein demonstrated that mesenchymal stem cells (MSCs) are locally present in the bone marrow of adults (Friedenstein et al. 1966). More recently, it became clear that most specialized tissues in the body contain a local pool of stem- or progenitor cells. Local stem- or progenitor cells can be derived from all musculoskeletal tissues and show quite some resemblances to bone marrow derived MSCs (BMSCs) (Sakaguchi et al. 2005; Yoshimura et al. 2007; Segawa et al. 2009). A multitude of nomenclature to denote these cells is being used in literature. For this chapter we will refer to them as local Mesenchymal Progenitor Cells (MPCs) that are present in the tissues of the musculoskeletal system.

Different properties and functions can be appointed to MPCs. To review them we will subsequently describe the following aspects:

- Derivation: overview of MPCs derived from various musculoskeletal tissues.
- Characteristics and properties: markers, yield and proliferation, ageing and senescence.
- Regenerative capacity: differentiation of MPCs and the secretion of trophic factors that can influence tissue regeneration.
- Potential applications: in vivo animal and clinical results regarding tissue regeneration and other applications.

17.2 Derivation

The musculoskeletal system consists of many different tissues. MPCs have been isolated from the following mesenchymal tissues: bone marrow (Pittenger et al. 1999), adipose tissue (Zuk et al. 2002), muscle (Burdzinska et al. 2008), cartilage (Tallheden et al. 2006), synovial membrane or -fluid (Morito et al. 2008; Fan et al. 2009), periosteum/perichondrium (Upton et al. 1981; Hutmacher and Sittinger 2003), tendons/ligaments (Bi et al. 2007; Singhatanadgit et al. 2009) and bone

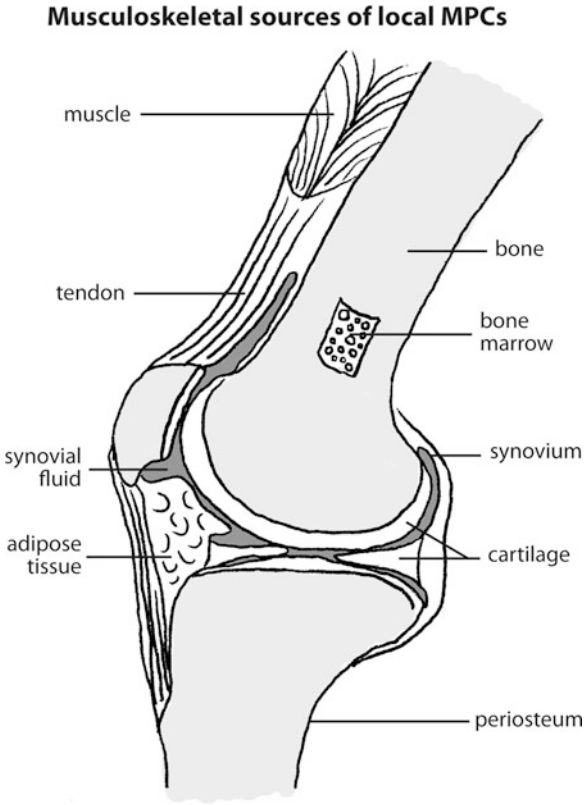


Fig. 17.1 Local mesenchymal progenitor cells (MPCs) can be derived from all these musculoskeltal tissues. Ligaments and perichondrium are not shown in this image, but contain MPCs as well

(Sakaguchi et al. 2004) (Fig. 17.1). Cells derived from these various tissues have different specific characteristics and capacities, but they also display many similarities. The main common feature of these various cell populations is that they all have the potential to differentiate into multiple mesodermal lineages. A brief description of the various tissue sources for MPCs is given below.

17.2.1 Bone Marrow

BMSCs are the most extensively studied musculoskeletal MPCs. BMSCs are non-hematopoietic cells that reside in the bone marrow. They were first described by Friedenstein et al., as clonal, plastic adherent cells, functioning as a source of the osteoblastic, adipogenic and chondrogenic cell lineages (Friedenstein et al. 1976). BMSCs play an important role in the bone marrow's microenvironment (Bobis et al. 2006). The main function of MSCs in the bone marrow is to create a tissue framework, serving as mechanical support for the hematopoietic cell system. For further specifics on BMSCs we refer to the previous chapter where BMSCs are extensively discussed.

17.2.2 Adipose Tissue

Adipose tissue is derived from the mesenchyme and contains a supportive stroma. Zuk et al. were the first to report the isolation of multi-potent stem cells from this stromal fraction of adipose tissue (Zuk et al. 2001). We would like to mention specifically the possibility to harvest local fat from fatpads in the joint. These structures have been demonstrated to contain MPCs (Dragoo et al. 2003; Wickham et al. 2003). A clear advantage of this cell population is their ready accessibility and the excellent availability of large quantities of tissue that can be harvested. Also for further specifics of this cell population we refer to the previous chapter.

17.2.3 Muscle

Roughly two MPC groups can be found in adult muscle tissue: satellite cells (which are considered uni-potent stem cells) and multi-potent muscle-derived stem cells. Satellite cells were first described in 1963. Satellite cells comprise a heterogenous cell population that resides under the basal lamina which surrounds muscle fibres (Mauro 1961). Within this heterogenous cell population a Pax7 positive sub-population can be identified which is called the "typical" satellite cell (Seale et al. 2000). Adult muscle satellite cells can give rise to transient amplifying cells (progenitors) and myoblasts. These myoblasts fuse with myofibres and play a principal role in postnatal skeletal muscle growth and regeneration (Sacco et al. 2008). The multi-potent muscle-derived stem cells (also referred to as skeletal muscle side population) have been discovered more recently. These cells comprise a more homogenous cell population that is less abundant in adult muscle (Qu-Petersen et al. 2002). These cells play a role during muscle homeostasis and regeneration. While they do posses multi-lineage differentiation potential, these skeletal muscle side population cells display a distinct preference for myogenic differentiation.

17.2.4 Cartilage

Articular cartilage is an avascular, aneural tissue of a stiff but compressible nature. The density of cells present in cartilage is very low, and cell mobility is limited through the surrounding matrix. The lack of vascularisation and innervation together with a low cell density and the relative immobility of the cells in cartilage, are the reasons why cartilage has a very low capacity for self-repair (Tallheden et al. 2006). In general, cartilage defects caused by trauma or mechanical wear, tend to further degenerate instead of regenerate. Various studies have shown that the superficial zone of articular cartilage regulates cartilage development and growth (Hayes et al. 2001; Hunziker et al. 2007). The first report describing isolation of a population of stem/

progenitor cells from the superficial zone dates from 2004 (Dowthwaite et al. 2004). Recently, Koelling et al. reported on the isolation of a population of progenitor cells from repair tissue of late stage human osteoarthritic cartilage which could not be isolated from healthy cartilage (Koelling et al. 2009). These cells possessed characteristics of clonogenicity, multipotency and migratory activity. Although most studies regarding cartilage derived progenitor cells comprise articular cartilage, other cartilage types such as meniscus (Segawa et al. 2009) and intervertebral discs (Risbud et al. 2007; Feng et al. 2010) contain progenitor cells as well.

17.2.5 Synovium and Synovial Fluid

Cells present in the synovium produce synovial fluid that functions as a joint lubricant. The synovium is a thin (two to three cell layers thick) membrane lining the non-articular joint surfaces, thereby providing a synovial fluid-filled cavity around the cartilage (Fan et al. 2009). De Bari et al. were the first to report successful isolation of stem cells from synovial membrane in 2001 (De Bari et al. 2001). Various names have been used for describing stem- or progenitor cells derived from the synovium, including synovium-derived stem cells, synovium-derived MSCs, and synovial progenitor cells. Interestingly, synovial MPCs can be generated from healthy synovium but also from rheumatoid- and osteoarthritis patients (Zimmermann et al. 2001; Nagase et al. 2008).

The number of MPC/stem cells in synovial fluid increases after trauma and these cells have been hypothesized to be important in repair of intra-articular structures after injury. For instance, the number of colony forming cells in synovial fluid was reported to be a 100-fold higher in knees several weeks after injury of the anterior cruciate ligament than in knees from healthy volunteers (Morito et al. 2008). These cells are likely to originate from the synovial membrane, since there is a positive correlation between intra-articular synovial fragments and the number of MPCs in synovial fluid (Jones et al. 2008). Moreover, MPCs locally present in the synovium were found to proliferate in response to damage in an animal OA model (Kurth et al. 2011). The injured tissues can attract MSC from this source to the synovial fluid by the secretion of cytokines and chemokines such as stromal cell-derived facor-1 (SDF-1), CXCR4 or VEGF (Spaeth et al. 2008).

17.2.6 Periosteum and Perichondrium

These cells are amongst the first musculoskeletal tissues that have been used to regenerate bone and cartilage. The first report describing the osteogenic function of periosteum dates from 1742 (Gysel 1983) and Fell et al. were the first to describe culturing and isolation of periosteum cells in 1932 (Fell 1932). Periosteum as well as perichondrium contains a so-called cambium layer, which is attached to the bone and cartilage respectively. This cambium layer contains cells, capable of

proliferating in order to form new tissue. The periosteum and perichondrium plays a role in cartilage or bone remodelling during the skeletal growth period and during repair after wounding.

17.2.7 Tendon and Ligament

In their biological function, tendons and ligaments mainly transmit tensile forces. Per definition, tendons form the link between a muscle belly and a bone, while ligaments attach bone to bone (Rumian et al. 2007). Ligaments are composed of the same basic components as tendons, although differences do exist to provide the specific mechanical properties needed for their tasks. For instance, the collagen fibrils in ligaments are not uniformly parallel oriented, in order to allow for multiaxial loading patterns (Rumian et al. 2007). The primary unit of a tendon or a ligament is the collagen fiber. This extracellular matrix is produced by the tissue cells that lie between the collagen fibers (Kannus 2000). An intriguing feature of tendon is its possible plasticity. Conversion of tendon into cartilage has been observed as a consequence of (non)surgical trauma (Rooney et al. 1993; McClure 1983). Various tendons and ligaments, including cruciate ligaments, periodontal ligaments and patellar- and hamstring tendons, have been demonstrated to contain stem/progenitor cells (de Mos et al. 2007; Scutt et al. 2008; Singhatanadgit et al. 2009).

17.2.8 Bone

Bone and bone marrow are physically virtually co-localized. Local MPCs have been demonstrated to grow out of trabecular bone fragments, either with or without enzymatically digesting the fragments (Noth et al. 2002; Sanchez-Guijo et al. 2009). Since the close anatomical relationship between these cells and BMSCs, a definitive statement about whether these cells arise from a different source is difficult to make. Furthermore, MPCs derived from trabecular bone become virtually identical to bone marrow derived MSCs upon subcultivation for approximately two passages (Sakaguchi et al. 2004). Therefore, these cells will not be discussed separately here.

17.3 Characteristics and Properties

17.3.1 Cell Markers

Today it is unavoidable to characterize the obtained cells by fluorescence activated cell sorting (FACS) analyses. This technique is introduced from the field of haematology where research on stem and progenitor cells is further advanced. In contrast to the

haematology, unique markers for mesenchymal and progenitor cells are not yet available (Jones and McGonagle 2008). Therefore a large set of different markers is used. Virtually all described MPC populations used in research are heterogeneous groups of cells, attributing to different characterisations by different investigators. According to the International Society for Cellular Therapy (Alessandri et al. 2004), a multipotent stromal cell is defined by the following criteria (a) its property of adherence to plastic; (b) its phenotype: negative for: CD14 or CD11b, CD19 or CD79a, CD34, CD45, HLA-DR, and positive for CD73, CD90, CD105; and (c) its capacity to be differentiated into three lineages, chondrocyte, osteoblast, and adipocyte. Criteria (a) and (c) are met by MPCs derived from all tissues discussed in this chapter. These topics will be further dealt with later in this section. Regarding cell phenotype, our literature review showed that MPCs derived from the previously mentioned tissues are negative for CD31, CD34, CD45 and CD117. They are reported to be positive for CD44, CD90, CD105, CD146, CD166 and STRO-1 (Young et al. 2001; Fickert et al. 2003, 2004; Alessandri et al. 2004; Sakaguchi et al. 2005; Giurea et al. 2006; Shirasawa et al. 2006; Bi et al. 2007; de Mos et al. 2007; Burdzinska et al. 2008; Jones et al. 2008; Nesti et al. 2008; Sacco et al. 2008; Scutt et al. 2008; Fan et al. 2009; Grogan et al. 2009; Wada et al. 2009). Although this combination of positive and negative markers is quite comparable for markers generally accepted for bone marrow- and adipose tissue derived MSCs, differences between the two latter cell populations and MPCs derived from other mesenchymal tissues have been reported (Sakaguchi et al. 2005; Shirasawa et al. 2006; Yoshimura et al. 2007; Koga et al. 2008; Segawa et al. 2009). Most frequently reported difference between these cells is differentiation capacity, which will be discussed later in this chapter. Some groups have described a so-called “side population (SP)” of progenitor cells. These cells, originally described as hematopoietic stem cells, have a unique FACS profile after staining with Hoechst 33342 dye. They can not only be obtained from bone marrow but from other tissues as well, including synovium and muscle (Liadaki et al. 2005; Teramura et al. 2008). Transcriptional profiles for SP and the more differentiated non-SP cells appear to be different. Amongst the genes upregulated in SP cells are genes that implicate the quiescent status of the cells, maintenance pluripotency and the capacity to undergo asymmetric division (Rochon et al. 2006). Some groups claim that these cells have superior properties in comparison to the remaining “main population” of cells, but this topic is too specific to include in this chapter.

17.3.2 Cell Yield: Isolation and Proliferation

The common protocol for isolation of MPCs, irrespective of tissue source, is mincing the tissue followed by enzymatic digestion. The enzyme collagenase is mostly used for this purpose, sometimes in combination with other enzymes like dispase. The duration of digestion and enzyme dose is variable, but in general lower collagen content of tissues results in a shorter isolation protocol. After tissue digestion, MPCs

are in general selected upon plastic adherence, which is in accordance to BMSC isolation (Dominici et al. 2006). This doesn't hold true for muscle satellite cells or cartilage derived MPCs. These cells are selected using vitrogen gel or fibronectin adherence respectively (Yablonka-Reuveni et al. 1999; Dowthwaite et al. 2004). However, it is to be expected that these generally applied MPC isolation protocols are merely empirically based and not necessarily optimized. Changing concentration, duration and composition of enzymes might further increase cell yields as well as their viability.

Occasionally, cells are isolated by cutting the tissue in small pieces for cells to grow out. This is often done if the amount of tissue is too limited to obtain sufficient cell number after enzymatic digestion. Results of these studies demonstrate that for bone, perichondrium and tendon it is indeed possible to obtain cells with multilineage capacity this way (Van Osch et al. 2000; Noth et al. 2002; de Mos et al. 2007).

For isolation of MPC from bone marrow aspirates a different protocol is followed. These cells are not isolated enzymatically but solely selected by adherence to tissue culture plastic after plating the biopsy. Sometimes, this is preceded by lyses of the red blood cells and/or a density gradient centrifugation to select the mononuclear fraction.

Cell yields from different tissues vary per isolation source and also depend on the original cellularity of the tissue. Sakaguchi et al. found comparable colony forming units (CFU) per 10^3 nucleated cells for adipose, synovium-, periosteum- and muscle derived MPCs (Sakaguchi et al. 2005). Bone marrow showed less CFU per 10^3 nucleated cells, but this was compensated by a higher number of nucleated cells per tissue volume. Others report roughly comparable results with a tendency towards higher colony forming units per nucleated cells obtained from synovium compared to periosteum, muscle and cruciate ligaments (Yoshimura et al. 2007; Segawa et al. 2009). No unambiguous differences regarding the proliferation of the isolated cell populations could be observed up to passage eight, although synovial derived cells showed a trend towards higher proliferation (Sakaguchi et al. 2005; Yoshimura et al. 2007). Overall, cell yields from these tissues and proliferation capacities of the isolated cells appear to be within the same order of magnitude. Therefore, the ease of harvesting sufficient amounts of tissue might become of more importance for selection of the most suitable cell source in future clinical practice.

17.3.3 Ageing and Senescence

To evaluate the possibilities to prevent or treat musculoskeletal disorders using autologous cell-based therapies, it is important to know how the number and function of MPC in musculoskeletal tissues are affected by age and/or disease. In the ageing organism, regenerative capacities of tissues tend to decrease (Brooks and Faulkner 1990; Kasper et al. 2009). Possible explanations for this declining regenerative capacity could be an age-related change in numbers or features of MPCs (cell intrinsic factors) or modifications in the surrounding environment (cell extrinsic

factors) (Burdzinska et al. 2008). These alterations have not been fully elucidated yet, and literature about this subject is sometimes contradictory.

In bone marrow for instance, a reduction of colony-forming efficiency was found with increasing age using donors ranging from infants to the age of 60 years (Caplan et al. 1998). Scharstuhl et al. on the other hand, found no correlation between age and the number of mononuclear cells in bone marrow, BMSC yield, cell size, proliferative capacity or cellular spectrum of the harvested cells from adult human donors (Scharstuhl et al. 2007). Similarly, for periosteum derived MPCs opposing results have been published regarding the influence of age. Both donor age dependent (Nakase et al. 1993) and independent (Koshihara et al. 1989) effects on osteochondrogenic potential of periosteum MPCs have been reported. Periosteal cells are reported to maintain osteochondrogenic potential up to ten population doublings. This potential eventually diminishes upon further passaging (Nakahara et al. 1991).

Muscle tissue is the last tissue for which inconsistencies are described regarding age-related findings. The number of progenitor cells in muscles has been reported to decrease, remain constant or increase with rising age (Brack and Rando 2007). Other cell intrinsic factors like telomere shortening and increased tendency to undergo apoptosis play a role in declining muscle regeneration upon ageing too (Brack and Rando 2007). Cell extrinsic factors that determine appropriate activation and efficient proliferation before terminal differentiation, are also proposed to play a key role in regenerating capacities of ageing muscle tissue. This is explained here with some examples. In aged muscle, progenitor cells prematurely shift from a proliferation phase to a differentiated state due to alterations of Wnt and Notch signaling (Conboy et al. 2003; Brack et al. 2007). This leads to less regeneration capability in aging organisms. Interestingly, this trend appears to be reversible. When aged satellite cells are exposed to a young systemic environment, Notch activation is re-established again (Conboy et al. 2005). Another known feature of satellite cells is the tendency to convert to fibroblasts *in vitro* upon increasing age. Exposure of aged cells to serum derived from young animals reduces this tendency, further emphasizing the role of extrinsic factors in (aged) tissue regeneration (Conboy et al. 2005).

Cartilage derived chondroprogenitor cells are described to have a relative high telomerase activity (Khan et al. 2009). Telomerase, first described by Greider et al., prevents telomere shortening during cell division (Greider and Blackburn 1985). By doing so, this enzyme postpones cell senescence and increases the maximum amount of cell doublings a cell can undergo. Cartilage derived progenitors undergo a high number of initial population doublings before a plateau is reached upon approximately 50 population doublings. After approximately 25 population doublings telomerase activity appears to decrease, leading to morphological and functional cell senescence (Khan et al. 2009).

Synovium MPCs have also been described to have limited senescence and can be expanded *in vitro* to large numbers. Their proliferative capacity doesn't appear to be affected by donor age. However, despite their high proliferative capacity, synovium MPCs have undetectable telomerase activity (De Bari et al. 2001). Their multipotent capacity is not influenced by donor age, cell passages or cryopreservation (De Bari et al. 2001).

In summary, the fact that regenerative capacities of ageing tissues tend to decrease is not entirely understood. However, it is clear that both cell intrinsic and cell extrinsic properties play a role in this process. In order to evaluate the applicability of cell-based therapies in musculoskeletal disorders, animal and clinical studies are a prerequisite. Results regarding this topic are mentioned in the "Potential applications for therapies" section.

17.4 Regenerative Capacity

17.4.1 Differentiation

MPCs derived from the various described musculoskeletal tissues have all been shown to have a multilineage differentiation potential (Sakaguchi et al. 2005; Thornemo et al. 2005; Segawa et al. 2009). This means they all have been shown to be able to differentiate into multiple mesodermal lineages including the osteogenic, adipogenic and chondrogenic lineage. The only exception is the "typical" satellite cell, which is generally considered a unipotent myogenic stem cell (Burdzinska et al. 2008). Although the latter may be regarded as a myoprogenitor cell, it does have true stem cell properties including self-renewal by asymmetric division (Kuang et al. 2007). Although cells derived from these previously mentioned tissues have the capacity to differentiate into different lineages, they all show a preference, in general for differentiating towards the tissue they were originally derived from. So, cartilage derived MPCs show a tendency towards chondrogenic differentiation (Hattori et al. 2007) and muscle derived MPCs towards the myogenic lineage (Muskiewicz et al. 2005). Since no differentiation protocols are available for differentiating cells in vitro towards a tendon, synovial or periosteal lineage for instance, cells derived from these tissues commonly are differentiated into adipocytes, osteocytes and chondrocytes as well to prove multilineage differentiation potential. Periosteal MPCs have great osteogenic potential (Hutmacher and Sittinger 2003) whereas synovium derived MPCs have a preference for the chondrogenic lineage (Mochizuki et al. 2006). Nevertheless, it is very difficult to state that local progenitors from one type of tissue are more suitable to form a certain tissue than other MPCs. Due to the fact that virtually all cells discussed in this chapter comprise heterogeneous groups of cells, it is hard to tell whether differentiation preferences arise from specific stem cell related features or are simply a consequence of differences in the presence of local (further differentiated) progenitors. Furthermore, differentiation studies *in vitro* are not directly translatable to an *in vivo* situation. MPCs are exposed to specific local (micro)environments during tissue development as well as during the mature stage. When *in vivo* applied, it seems logical that these cells can respond different to tissue specific growth factors or other stimuli. More information about the microenvironments of the various MPCs is necessary, together with knowledge about the heterogeneity of cell populations. An increasing number of clonal studies using single cells to show true multilineage differentiation and/or

self-renewal of different MPCs are reported (Barbero et al. 2003; Fujii et al. 2008; Sacco et al. 2008; Singhatanadgit et al. 2009). Direct comparison of clonal cells derived from different musculoskeletal tissues should be a focus of future research in order to form a founded opinion about optimal cell sources.

Besides differentiating into mesodermal lineages, bone marrow stromal derived cells have been shown to be able to give rise to hepatic cells (endodermal lineage) and mature astrocytes and neurons (ectodermal lineage) as well (Tomita et al. 2006; Oh et al. 2007). This process, where a stem cell differentiates into cell types from a different germ layer than the one it originally resided in, is termed transdifferentiation. Although this has not been extensively investigated for the MPCs discussed in this chapter, muscle derived MPCs for example have been demonstrated to be able to transdifferentiate into endo- and ectodermal lineages as well (Schultz et al. 2006). This might suggest that MPCs from other sources might also have transdifferentiation capacity, which has to be shown in future investigations.

17.4.2 Trophic Factors

MPCs can contribute to tissue repair by differentiating into a mature tissue cell and forming extracellular matrix to repair damaged tissue. In addition these cells contribute to tissue repair: by the production of trophic factors. These trophic factors are capable of attracting (more) stem cells to the damaged area and they can have an immunomodulatory effect (Caplan and Dennis 2006). For bone marrow and adipose derived cells there is a fast increasing amount of information on trophic factors (Jones and McTaggart 2008; Siegel et al. 2009). MPCs derived from bone marrow and adipose tissue, but also MPCs from periodontal ligament, have been shown to have a suppressive effect upon peripheral blood mononuclear cell proliferation (Wada et al. 2009). This suppressive effect was found both in mixed lymphocyte reactions (allowing cell-cell contact) and in transwell co-cultures (avoiding direct contact). The latter emphasizes the paracrine effects of these cells.

17.5 Potential Applications for Therapies

Huge potential resides in the MPCs discussed in this chapter regarding musculoskeletal tissue regeneration, either by differentiating into more mature tissue cells or by their modulatory properties. The potential of MPCs for tissue regeneration or immune modulation is demonstrated by the huge amount of clinical trials that are already performed or are still ongoing, using bone marrow derived MSCs (Bobis et al. 2006; Giordano et al. 2007). The application of these cells encompasses various fields, including cardiovascular diseases, osteogenesis imperfecta, graft versus host disease and neurological disorders like amyotrophic lateral sclerosis, etc. (Giordano et al. 2007; Jones and McGonagle 2008).

Clinical studies or applications with musculoskeletal stem cells generated from other tissues than bone marrow have been performed with muscle derived MPCs, periosteum and perichondrium. Muscle MPCs have been used in clinical trials for treating myocardial ischemia (Joggerst and Hatzopoulos 2009). Periosteum has proven it's added value in treating bone defects (Schmelzeisen et al. 2003; Yamamiya et al. 2008) and has been used as a therapy for local cartilage defects for more than two decades (Niedermann et al. 1985; Alfredson et al. 1999). Perichondrium has been applied as a graft to treat isolated chondral defects (Homminga et al. 1990; Bouwmeester et al. 2002). Although MPCs likely play a role in these studies, tissue transplants were used rather than isolated cells. Therefore, we cannot state that the positive results were solely based on the regenerative capacities of transplanted MPCs. Furthermore, although promising results were obtained using periosteum for treating bone defects, golden standard still is the use of autologous bone grafts. Regarding local cartilage defects, subchondral drilling and autologous chondrocyte implantation (see later) have become the main therapies. Animal experiments with MPCs of other musculoskeletal tissues show hopeful results. Muscle MPCs have been reported to contribute to up to 94 % of myofibers after intramuscular injection into dystrophic mice (Cerletti et al. 2008). Furthermore, satellite cells have been shown to be able to undergo approximately 14–17 cell doublings after single cell transplantation in vivo (Sacco et al. 2008). A possible drawback of muscle MPCs is their low migrating capacities requiring local delivery, although homing of these cells into damaged muscle after intravenous injection has been reported (Muskiewicz et al. 2005). Synovial MPCs in their turn have proven in vivo to contribute to cartilage repair (Pei et al. 2009), meniscal regeneration (Horie et al. 2009), muscle repair (De Bari et al. 2003) and to accelerate remodeling of tendon to bone healing in a bone tunnel model (Ju et al. 2008). Lastly, tendon- and ligament derived MPCs can play a role in regenerating damaged tendon and ligament respectively (Gronthos et al. 2006; Kryger et al. 2007). Although cartilage has been shown to contain progenitor cells, applications using only these MPCs do not yet exist. In the field of cartilage repair, autologous chondrocyte implantation is a well established, cell-based and clinically applied cartilage repair technique (Brittberg et al. 1994). This technique however uses predominantly differentiated chondrocytes to fill the cartilage defect, thereby not meeting the definition of stem cell therapy. The contribution of cartilage MPC to the outcome of ACI is not known and would be an interesting research question.

In summary, the applicability of MPCs of a certain tissue for an application will depend on ease of harvesting and the ability to control function. At the moment, further evidence has to come from properly organized and controlled animal as well as clinical studies.

The variability of the outcome of different studies where MPCs are used is of great concern. Part of the variation might be due to variation in MPC characteristics between donors. Better characterisation of the cells or selection of cells before use can improve outcome. The first study with cell selection (although this did not involve MPCs), for regenerative medicine of the musculoskeletal system has been performed in cartilage repair (Saris et al. 2008). In addition to the variation in MPC characteristics, variability in outcome of clinical studies will also be caused by the differences in the host environment where the cells are introduced. Stage of disease,

but also general patient characteristics like BMI, age and gender will determine MPC fate and function (Caplan et al. 1998). How these factors influence MPCs is largely unknown at the moment. It is important to take this into account when designing clinical studies.

Instead of actually applying or injecting MPCs for musculoskeletal disorders, some therapies aim at stimulating or attracting stem cells to the damaged tissue. Some examples of this approach are shockwave therapy, pulsed electromagnetic fields (PEMF) or the use of cell attracting growth factors. These strategies are being investigated as possible therapies for osteoporosis, bone non-unions, osteochondral defects and cartilage regeneration (Wang et al. 2009; van Bergen et al. 2009; van der Jagt et al. 2009; Lee et al. 2010).

17.5.1 Tumour Formation

Although formation of tumours after the use of adult MPCs have never been reported, the capacities of MPC are not yet fully investigated and understood. The role of BMSCs in carcinogenesis is a relative new feature. BMSCs have a distinct homing potential to a wide range of organs after systemic administration (Koc et al. 2000; Gao et al. 2001; Devine et al. 2003). The ability of BMSCs to home to primary tumour sites and metastases has been demonstrated by several studies (Nakamura et al. 2004; Khakoo et al. 2006; Kucerova et al. 2007). Their role at these tumour sites and their potential effect on tumour development can be bivalent. Both pro- and anti-proliferative effects of BMSCs have been reported regarding this aspect (reviewed in Lazennec and Jorgensen 2008). Possible explanations for these contradictory findings might be the immunomodulatory effects of BMSCs, which can be both in favour and to the detriment of tumour development. Another factor might be the excretion of VEGF by BMSCs, which plays a role in (neo)vascularisation.

The effect or role of the MPCs in tumour formation or -growth has not been investigated so far. It is a known fact that cells can undergo karyotypic changes upon (long-term) culturing in vitro (Wahrman et al. 1984; Bochkov et al. 2007). Cell based regenerative therapies have to be proceeded cautiously, and these aspects certainly have to be investigated before proceeding to large-scale clinical translation.

17.6 Conclusions, Discussion and Future Development in Research

It is clear that local progenitor cells can be isolated from various tissues in the musculoskeletal system and that these cells can play a role in tissue repair. Overall, cell yields from the different tissues, proliferation capacities and cell membrane markers of the isolated cells appear to be similar. Cells derived from the various musculoskeletal tissues have all been shown to have a multilineage differentiation potential. Besides differentiating into a mature tissue cell, secretion of trophic factors

is assigned a possible function of stem cell that might be important in tissue repair. Musculoskeletal progenitor cells posses a huge capacity for application in regenerative medicine. Table 17.1 summarizes various features of MPCs derived from the tissues discussed in this chapter.

It is not always straightforward to determine whether the isolated MPCs are really local cells, especially in damaged tissues. Tissue damage has been demonstrated to attract stem cells from the circulation. These cells are recruited from the bone marrow and home in the damaged tissue to support repair (Sordi 2009). These recruited cells, once isolated from damaged tissue, might therefore be mistaken for local MPCs. Furthermore, the tissues of the musculoskeletal system, with exception of cartilage and tendon, are well vascularised. These small vessels contain pericytes. A pericyte is a relatively undifferentiated cell, which serves to support small vessels. They were first described in the nineteenth century as cells located between the endothelial cells and the parenchymal cells in capillaries and post capillary venules. Apart from their role in blood vessel formation, pericytes were recognized to contribute to bone formation (Schor et al. 1995) and they might play an important role in tissue repair and regeneration in many musculoskeletal tissues. Pericytes have multilineage differentiation capacity too, and there could be a chance that these cells are very closely related to the local progenitors described in this chapter. However, only recently they regained a lot of interest as adult progenitor cells with multilineage capacity and methods to isolate and purify these cells are being developed (Crisan et al. 2008). Especially in well vascularised tissues, such as bone marrow, adipose tissue and muscle, the perivascular cells might be regarded as local MPC. Further research will have to elucidate the role of local stem cells as well as pericytes and systemic stem cells present in the circulation, in intrinsic tissue repair capacity as well as their use in cell therapy.

For cell therapeutic application optimal isolation and culture conditions for each cell type has to be found and tailored for every specific application. Choice for optimal cell type to regenerate a tissue might very likely depend on criteria related to ease of harvesting. In this respect, bone marrow and adipose tissue are attractive candidates for harvesting large amounts of cells in a relatively easy procedure.

The various musculoskeletal stem cells all have multilineage differentiation capacity. Although this offers interesting opportunities, it also emphasizes the importance of learning to control and direct differentiation and tissue formation by these cells to prevent undesired tissue- or tumour formation. Better characterisation of musculoskeletal stem cells and more knowledge about lineage differentiation is required to fully understand the potential of each individual source of cells for each of the different applications. In this respect, not only differentiation into mature tissue cells, but also the secretion of trophic factors deserves more study. Furthermore the fate of the cells after application in-vivo has to be studied with modern imaging techniques both in animal and in human patients (Srinivas et al. 2010) to be able to answer the questions: Where do they home?; How long do they stay viable?; What is their activity? Finally there is a need for more understanding of how characteristics of host microenvironment influence fate and function of the cells in order to optimize the results of cell therapy in musculoskeletal disorders.

Table 17.1 Overview of various features of MPCs derived from different tissues

Tissue source	Regenerative capacity shown	Clonal multilineage potential shown	Clinical studies performed	Availability/ accessability[a]
Bone marrow	Differentiation and trophic factors	Yes	Yes	+/±
Adipose tissue, incl. intra-articular fat pads	Differentiation and trophic factors	Yes	Yes	+/+
Muscle	Differentiation	Yes	Yes	+/+
Cartilage	Differentiation	Yes	Performed with cartilage derived cells, not specific MPCs	–/–
Synovium and synovial fluid	Differentiation	No	No	±/±
Periosteum and perichodrium	Differentiation	No	Performed with whole tissue, not isolated cells	–/–
Tendon and ligament	Differentiation and trophic factors	Yes	No	±/–[b]

[a]These are arbitrary measures. +, ± and – represents a good, medium and limited availability (i.e. amount of obtainable tissue) and accessibility (i.e. invasiveness of procedure needed to harvest the tissue) respectively

[b]In maxillofacial surgery, the periodontal ligament is readily available and accessible. However, in limbs this cell source is less abundant and accessible

Acknowledgements The authors are supported by the Dutch Platform for Tissue Engineering; the Dutch Arthritis Association; the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science; the ENCITE consortium of the European Community under the 7th framework programme; and REMEDIC, a Network on Regenerative Medicine of the European Science Foundation. We would like to thank Monique Bernsen for critical reading the text and Ton Everaers for his assistance with creating the figure.

References

Alessandri G, Pagano S, Bez A, Benetti A, Pozzi S, Iannolo G et al (2004) Isolation and culture of human muscle-derived stem cells able to differentiate into myogenic and neurogenic cell lineages. Lancet 364(9448):1872–1883

Alfredson H, Thorsen K, Lorentzon R (1999) Treatment of tear of the anterior cruciate ligament combined with localised deep cartilage defects in the knee with ligament reconstruction and autologous periosteum transplantation. Knee Surg Sports Traumatol Arthrosc 7(2):69–74

Barbero A, Ploegert S, Heberer M, Martin I (2003) Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum 48(5):1315–1325

Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W et al (2007) Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nat Med 13(10):1219–1227

Bobis S, Jarocha D, Majka M (2006) Mesenchymal stem cells: characteristics and clinical applications. Folia Histochem Cytobiol 44(4):215–230

Bochkov NP, Voronina ES, Kosyakova NV, Liehr T, Rzhaninova AA, Katosova LD et al (2007) Chromosome variability of human multipotent mesenchymal stromal cells. Bull Exp Biol Med 143(1):122–126

Bouwmeester PS, Kuijer R, Homminga GN, Bulstra SK, Geesink RG (2002) A retrospective analysis of two independent prospective cartilage repair studies: autogenous perichondrial grafting versus subchondral drilling 10 years post-surgery. J Orthop Res 20(2):267–273

Brack AS, Rando TA (2007) Intrinsic changes and extrinsic influences of myogenic stem cell function during aging. Stem Cell Rev 3(3):226–237

Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C et al (2007) Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science 317(5839):807–810

Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 331(14):889–895

Brockes JP, Kumar A (2002) Plasticity and reprogramming of differentiated cells in amphibian regeneration. Nat Rev Mol Cell Biol 3(8):566–574

Brooks SV, Faulkner JA (1990) Contraction-induced injury: recovery of skeletal muscles in young and old mice. Am J Physiol 258(3 Pt 1):C436–C442

Burdzinska A, Gala K, Paczek L (2008) Myogenic stem cells. Folia Histochem Cytobiol 46(4):401–412

Caplan AI, Dennis JE (2006) Mesenchymal stem cells as trophic mediators. J Cell Biochem 98(5):1076–1084

Caplan AI, Reuben D, Haynesworth SE (1998) Cell-based tissue engineering therapies: the influence of whole body physiology. Adv Drug Deliv Rev 33(1–2):3–14

Cerletti M, Jurga S, Witczak CA, Hirshman MF, Shadrach JL, Goodyear LJ et al (2008) Highly efficient, functional engraftment of skeletal muscle stem cells in dystrophic muscles. Cell 134(1):37–47

Conboy IM, Conboy MJ, Smythe GM, Rando TA (2003) Notch-mediated restoration of regenerative potential to aged muscle. Science 302(5650):1575–1577

Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA (2005) Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 433(7027):760–764

Crisan M, Deasy B, Gavina M, Zheng B, Huard J, Lazzari L et al (2008) Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myo-endothelial cells and pericytes. Methods Cell Biol 86:295–309

De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP (2001) Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum 44(8):1928–1942

De Bari C, Dell'Accio F, Vandenabeele F, Vermeesch JR, Raymackers JM, Luyten FP (2003) Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. J Cell Biol 160(6):909–918

de Mos M, Koevoet WJ, Jahr H, Verstegen MM, Heijboer MP, Kops N et al (2007) Intrinsic differentiation potential of adolescent human tendon tissue: an in-vitro cell differentiation study. BMC Musculoskelet Disord 8:16

Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R (2003) Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood 101(8):2999–3001

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8(4):315–317

Dowthwaite GP, Bishop JC, Redman SN, Khan IM, Rooney P, Evans DJ et al (2004) The surface of articular cartilage contains a progenitor cell population. J Cell Sci 117(Pt 6):889–897

Dragoo JL, Samimi B, Zhu M, Hame SL, Thomas BJ, Lieberman JR et al (2003) Tissue-engineered cartilage and bone using stem cells from human infrapatellar fat pads. J Bone Joint Surg Br 85(5):740–747

Fan J, Varshney RR, Ren L, Cai D, Wang DA (2009) Synovium-derived mesenchymal stem cells: a new cell source for musculoskeletal regeneration. Tissue Eng Part B Rev 15(1):75–86

Fell HB (1932) The osteogenic capacity in vitro of periosteum and endosteum isolated from the limb skeleton of fowl embryos and young chicks. J Anat 66(Pt 2):157–180, 11

Feng G, Yang X, Shang H, Marks IW, Shen FH, Katz A et al (2010) Multipotential differentiation of human anulus fibrosus cells: an in vitro study. J Bone Joint Surg Am 92(3):675–685

Fickert S, Fiedler J, Brenner RE (2003) Identification, quantification and isolation of mesenchymal progenitor cells from osteoarthritic synovium by fluorescence automated cell sorting. Osteoarthr Cartil 11(11):790–800

Fickert S, Fiedler J, Brenner RE (2004) Identification of subpopulations with characteristics of mesenchymal progenitor cells from human osteoarthritic cartilage using triple staining for cell surface markers. Arthritis Res Ther 6(5):R422–R432

Friedenstein AJ, Piatetzky S II, Petrakova KV (1966) Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 16(3):381–390

Friedenstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4(5):267–274

Fujii S, Maeda H, Wada N, Tomokiyo A, Saito M, Akamine A (2008) Investigating a clonal human periodontal ligament progenitor/stem cell line in vitro and in vivo. J Cell Physiol 215(3):743–749

Gao J, Dennis JE, Muzic RF, Lundberg M, Caplan AI (2001) The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs 169(1):12–20

Giordano A, Galderisi U, Marino IR (2007) From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells. J Cell Physiol 211(1):27–35

Giurea A, Ruger BM, Hollemann D, Yanagida G, Kotz R, Fischer MB (2006) STRO-1+ mesenchymal precursor cells located in synovial surface projections of patients with osteoarthritis. Osteoarthr Cartil 14(9):938–943

Greider CW, Blackburn EH (1985) Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 43(2 Pt 1):405–413

Grogan SP, Miyaki S, Asahara H, D'Lima DD, Lotz MK (2009) Mesenchymal progenitor cell markers in human articular cartilage: normal distribution and changes in osteoarthritis. Arthritis Res Ther 11(3):R85

Gronthos S, Mrozik K, Shi S, Bartold PM (2006) Ovine periodontal ligament stem cells: isolation, characterization, and differentiation potential. Calcif Tissue Int 79(5):310–317

Gysel C (1983) Henri-Louis Duhamel du Monceau (1700–1782–1982), growth and osteogenic function of the periosteum. Orthod Fr 54(2):605–621

Hattori S, Oxford C, Reddi AH (2007) Identification of superficial zone articular chondrocyte stem/progenitor cells. Biochem Biophys Res Commun 358(1):99–103

Hayes AJ, MacPherson S, Morrison H, Dowthwaite G, Archer CW (2001) The development of articular cartilage: evidence for an appositional growth mechanism. Anat Embryol (Berl) 203(6):469–479

Homminga GN, Bulstra SK, Bouwmeester PS, van der Linden AJ (1990) Perichondral grafting for cartilage lesions of the knee. J Bone Joint Surg Br 72(6):1003–1007

Horie M, Sekiya I, Muneta T, Ichinose S, Matsumoto K, Saito H et al (2009) Intra-articular injected synovial stem cells differentiate into meniscal cells directly and promote meniscal regeneration without mobilization to distant organs in rat massive meniscal defect. Stem Cells 27(4):878–887

Hunziker EB, Kapfinger E, Geiss J (2007) The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development. Osteoarthr Cartil 15(4):403–413

Hutmacher DW, Sittinger M (2003) Periosteal cells in bone tissue engineering. Tissue Eng 9(Suppl 1):S45–S64

Joggerst SJ, Hatzopoulos AK (2009) Stem cell therapy for cardiac repair: benefits and barriers. Expert Rev Mol Med 11:e20

Jones E, McGonagle D (2008) Human bone marrow mesenchymal stem cells in vivo. Rheumatology (Oxford) 47(2):126–131

Jones BJ, McTaggart SJ (2008) Immunosuppression by mesenchymal stromal cells: from culture to clinic. Exp Hematol 36(6):733–741

Jones EA, Crawford A, English A, Henshaw K, Mundy J, Corscadden D et al (2008) Synovial fluid mesenchymal stem cells in health and early osteoarthritis: detection and functional evaluation at the single-cell level. Arthritis Rheum 58(6):1731–1740

Ju YJ, Muneta T, Yoshimura H, Koga H, Sekiya I (2008) Synovial mesenchymal stem cells accelerate early remodeling of tendon-bone healing. Cell Tissue Res 332(3):469–478

Kannus P (2000) Structure of the tendon connective tissue. Scand J Med Sci Sports 10(6):312–320

Kasper G, Mao L, Geissler S, Draycheva A, Trippens J, Kuhnisch J et al (2009) Insights into mesenchymal stem cell aging: involvement of antioxidant defense and actin cytoskeleton. Stem Cells 27(6):1288–1297

Khakoo AY, Pati S, Anderson SA, Reid W, Elshal MF, Rovira II et al (2006) Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. J Exp Med 203(5):1235–1247

Khan IM, Bishop JC, Gilbert S, Archer CW (2009) Clonal chondroprogenitors maintain telomerase activity and Sox9 expression during extended monolayer culture and retain chondrogenic potential. Osteoarthr Cartil 17(4):518–528

Koc ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI et al (2000) Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol 18(2):307–316

Koelling S, Kruegel J, Irmer M, Path JR, Sadowski B, Miro X et al (2009) Migratory chondrogenic progenitor cells from repair tissue during the later stages of human osteoarthritis. Cell Stem Cell 4(4):324–335

Koga H, Muneta T, Nagase T, Nimura A, Ju YJ, Mochizuki T et al (2008) Comparison of mesenchymal tissues-derived stem cells for in vivo chondrogenesis: suitable conditions for cell therapy of cartilage defects in rabbit. Cell Tissue Res 333(2):207–215

Koshihara Y, Kawamura M, Endo S, Tsutsumi C, Kodama H, Oda H et al (1989) Establishment of human osteoblastic cells derived from periosteum in culture. In Vitro Cell Dev Biol 25(1):37–43

Kryger GS, Chong AK, Costa M, Pham H, Bates SJ, Chang J (2007) A comparison of tenocytes and mesenchymal stem cells for use in flexor tendon tissue engineering. J Hand Surg Am 32(5):597–605

Kuang S, Kuroda K, Le Grand F, Rudnicki MA (2007) Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell 129(5):999–1010

Kucerova L, Altanerova V, Matuskova M, Tyciakova S, Altaner C (2007) Adipose tissue-derived human mesenchymal stem cells mediated prodrug cancer gene therapy. Cancer Res 67(13):6304–6313

Kurth TB, Dell'accio F, Crouch V, Augello A, Sharpe PT, De Bari C (2011) Functional mesenchymal stem cell niches in the adult knee joint synovium in vivo. Arthritis Rheum 63(5):1289–1300

Lazennec G, Jorgensen C (2008) Concise review: adult multipotent stromal cells and cancer: risk or benefit? Stem Cells 26(6):1387–1394

Lee CH, Cook JL, Mendelson A, Moioli EK, Yao H, Mao JJ (2010) Regeneration of the articular surface of the rabbit synovial joint by cell homing: a proof of concept study. Lancet 376(9739):440–448

Liadaki K, Kho AT, Sanoudou D, Schienda J, Flint A, Beggs AH et al (2005) Side population cells isolated from different tissues share transcriptome signatures and express tissue-specific markers. Exp Cell Res 303(2):360–374

Lin NH, Gronthos S, Bartold PM (2008) Stem cells and periodontal regeneration. Aust Dent J 53(2):108–121

Mauro A (1961) Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 9:493–495

McClure J (1983) The effect of diphosphonates on heterotopic ossification in regenerating Achilles tendon of the mouse. J Pathol 139(4):419–430

Mochizuki T, Muneta T, Sakaguchi Y, Nimura A, Yokoyama A, Koga H et al (2006) Higher chondrogenic potential of fibrous synovium- and adipose synovium-derived cells compared with subcutaneous fat-derived cells: distinguishing properties of mesenchymal stem cells in humans. Arthritis Rheum 54(3):843–853

Morito T, Muneta T, Hara K, Ju YJ, Mochizuki T, Makino H et al (2008) Synovial fluid-derived mesenchymal stem cells increase after intra-articular ligament injury in humans. Rheumatology (Oxford) 47(8):1137–1143

Muskiewicz KR, Frank NY, Flint AF, Gussoni E (2005) Myogenic potential of muscle side and main population cells after intravenous injection into sub-lethally irradiated mdx mice. J Histochem Cytochem 53(7):861–873

Nagase T, Muneta T, Ju YJ, Hara K, Morito T, Koga H et al (2008) Analysis of the chondrogenic potential of human synovial stem cells according to harvest site and culture parameters in knees with medial compartment osteoarthritis. Arthritis Rheum 58(5):1389–1398

Nakahara H, Goldberg VM, Caplan AI (1991) Culture-expanded human periosteal-derived cells exhibit osteochondral potential in vivo. J Orthop Res 9(4):465–476

Nakamura K, Ito Y, Kawano Y, Kurozumi K, Kobune M, Tsuda H et al (2004) Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model. Gene Ther 11(14):1155–1164

Nakase T, Nakahara H, Iwasaki M, Kimura T, Kimata K, Watanabe K et al (1993) Clonal analysis for developmental potential of chick periosteum-derived cells: agar gel culture system. Biochem Biophys Res Commun 195(3):1422–1428

Nesti LJ, Jackson WM, Shanti RM, Koehler SM, Aragon AB, Bailey JR et al (2008) Differentiation potential of multipotent progenitor cells derived from war-traumatized muscle tissue. J Bone Joint Surg Am 90(11):2390–2398

Niedermann B, Boe S, Lauritzen J, Rubak JM (1985) Glued periosteal grafts in the knee. Acta Orthop Scand 56(6):457–460

Noth U, Osyczka AM, Tuli R, Hickok NJ, Danielson KG, Tuan RS (2002) Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells. J Orthop Res 20(5):1060–1069

Oh SH, Witek RP, Bae SH, Zheng D, Jung Y, Piscaglia AC et al (2007) Bone marrow-derived hepatic oval cells differentiate into hepatocytes in 2-acetylaminofluorene/partial hepatectomy-induced liver regeneration. Gastroenterology 132(3):1077–1087

Pei M, He F, Boyce BM, Kish VL (2009) Repair of full-thickness femoral condyle cartilage defects using allogeneic synovial cell-engineered tissue constructs. Osteoarthr Cartil 17(6):714–722

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284(5411):143–147

Qu-Petersen Z, Deasy B, Jankowski R, Ikezawa M, Cummins J, Pruchnic R et al (2002) Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J Cell Biol 157(5):851–864

Risbud MV, Guttapalli A, Tsai TT, Lee JY, Danielson KG, Vaccaro AR et al (2007) Evidence for skeletal progenitor cells in the degenerate human intervertebral disc. Spine (Phila Pa 1976) 32(23):2537–2544

Rochon C, Frouin V, Bortoli S, Giraud-Triboult K, Duverger V, Vaigot P et al (2006) Comparison of gene expression pattern in SP cell populations from four tissues to define common "stemness functions". Exp Cell Res 312(11):2074–2082

Rooney P, Walker D, Grant ME, McClure J (1993) Cartilage and bone formation in repairing Achilles tendons within diffusion chambers: evidence for tendon-cartilage and cartilage-bone conversion in vivo. J Pathol 169(3):375–381

Rumian AP, Wallace AL, Birch HL (2007) Tendons and ligaments are anatomically distinct but overlap in molecular and morphological features–a comparative study in an ovine model. J Orthop Res 25(4):458–464

Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM (2008) Self-renewal and expansion of single transplanted muscle stem cells. Nature 456(7221):502–506

Sakaguchi Y, Sekiya I, Yagishita K, Ichinose S, Shinomiya K, Muneta T (2004) Suspended cells from trabecular bone by collagenase digestion become virtually identical to mesenchymal stem cells obtained from marrow aspirates. Blood 104(9):2728–2735

Sakaguchi Y, Sekiya I, Yagishita K, Muneta T (2005) Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. Arthritis Rheum 52(8):2521–2529

Sanchez-Guijo FM, Blanco JF, Cruz G, Muntion S, Gomez M, Carrancio S et al (2009) Multiparametric comparison of mesenchymal stromal cells obtained from trabecular bone by using a novel isolation method with those obtained by iliac crest aspiration from the same subjects. Cell Tissue Res 336(3):501–507

Saris DB, Vanlauwe J, Victor J, Haspl M, Bohnsack M, Fortems Y et al (2008) Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture. Am J Sports Med 36(2):235–246

Scharstuhl A, Schewe B, Benz K, Gaissmaier C, Buhring HJ, Stoop R (2007) Chondrogenic potential of human adult mesenchymal stem cells is independent of age or osteoarthritis etiology. Stem Cells 25(12):3244–3251

Schmelzeisen R, Schimming R, Sittinger M (2003) Making bone: implant insertion into tissue-engineered bone for maxillary sinus floor augmentation-a preliminary report. J Craniomaxillofac Surg 31(1):34–39

Schor AM, Canfield AE, Sutton AB, Arciniegas E, Allen TD (1995) Pericyte differentiation. Clin Orthop Relat Res 313:81–91

Schultz SS, Abraham S, Lucas PA (2006) Stem cells isolated from adult rat muscle differentiate across all three dermal lineages. Wound Repair Regen 14(2):224–231

Scutt N, Rolf C, Scutt A (2008) Tissue specific characteristics of cells isolated from human and rat tendons and ligaments. J Orthop Surg 3:32

Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA (2000) Pax7 is required for the specification of myogenic satellite cells. Cell 102(6):777–786

Segawa Y, Muneta T, Makino H, Nimura A, Mochizuki T, Ju YJ et al (2009) Mesenchymal stem cells derived from synovium, meniscus, anterior cruciate ligament, and articular chondrocytes share similar gene expression profiles. J Orthop Res 27(4):435–441

Shirasawa S, Sekiya I, Sakaguchi Y, Yagishita K, Ichinose S, Muneta T (2006) In vitro chondrogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells. J Cell Biochem 97(1):84–97

Siegel G, Schafer R, Dazzi F (2009) The immunosuppressive properties of mesenchymal stem cells. Transplantation 87(9 Suppl):S45–S49

Singhatanadgit W, Donos N, Olsen I (2009) Isolation and characterisation of stem cell clones from adult human ligament. Tissue Eng Part A 15(9):2625–2636
Sordi V (2009) Mesenchymal stem cell homing capacity. Transplantation 87(9 Suppl):S42–S45
Spaeth E, Klopp A, Dembinski J, Andreeff M, Marini F (2008) Inflammation and tumor microenvironments: defining the migratory itinerary of mesenchymal stem cells. Gene Ther 15(10):730–738
Srinivas M, Aarntzen EH, Bulte JW, Oyen WJ, Heerschap A, de Vries IJ et al (2010) Imaging of cellular therapies. Adv Drug Deliv Rev 62(11):1080–1093
Tallheden T, Brittberg M, Peterson L, Lindahl A (2006) Human articular chondrocytes–plasticity and differentiation potential. Cells Tissues Organs 184(2):55–67
Teramura T, Fukuda K, Kurashimo S, Hosoi Y, Miki Y, Asada S et al (2008) Isolation and characterization of side population stem cells in articular synovial tissue. BMC Musculoskelet Disord 9:86
Thornemo M, Tallheden T, Sjogren Jansson E, Larsson A, Lovstedt K, Nannmark U et al (2005) Clonal populations of chondrocytes with progenitor properties identified within human articular cartilage. Cells Tissues Organs 180(3):141–150
Tomita M, Mori T, Maruyama K, Zahir T, Ward M, Umezawa A et al (2006) A comparison of neural differentiation and retinal transplantation with bone marrow-derived cells and retinal progenitor cells. Stem Cells 24(10):2270–2278
Upton J, Sohn SA, Glowacki J (1981) Neocartilage derived from transplanted perichondrium: what is it? Plast Reconstr Surg 68(2):166–174
van Bergen CJ, Blankevoort L, de Haan RJ, Sierevelt IN, Meuffels DE, d'Hooghe PR et al (2009) Pulsed electromagnetic fields after arthroscopic treatment for osteochondral defects of the talus: double-blind randomized controlled multicenter trial. BMC Musculoskelet Disord 10:83
van der Jagt OP, van der Linden JC, Schaden W, van Schie HT, Piscaer TM, Verhaar JA et al (2009) Unfocused extracorporeal shock wave therapy as potential treatment for osteoporosis. J Orthop Res 27(11):1528–1533
Van Osch GJ, Van Der Veen SW, Burger EH, Verwoerd-Verhoef HL (2000) Chondrogenic potential of in vitro multiplied rabbit perichondrium cells cultured in alginate beads in defined medium. Tissue Eng 6(4):321–330
Wada N, Menicanin D, Shi S, Bartold PM, Gronthos S (2009) Immunomodulatory properties of human periodontal ligament stem cells. J Cell Physiol 219(3):667–676
Wahrman MZ, Augenlicht LH, Higgins PJ (1984) Alterations in growth properties and cellular proteins of cloned hamster lung fibroblasts during long-term culture. Oncology 41(1):49–54
Wang CJ, Yang KD, Ko JY, Huang CC, Huang HY, Wang FS (2009) The effects of shockwave on bone healing and systemic concentrations of nitric oxide (NO), TGF-beta1, VEGF and BMP-2 in long bone non-unions. Nitric Oxide 20(4):298–303
Wickham MQ, Erickson GR, Gimble JM, Vail TP, Guilak F (2003) Multipotent stromal cells derived from the infrapatellar fat pad of the knee. Clin Orthop Relat Res 412:196–212
Yablonka-Reuveni Z, Seger R, Rivera AJ (1999) Fibroblast growth factor promotes recruitment of skeletal muscle satellite cells in young and old rats. J Histochem Cytochem 47(1):23–42
Yamamiya K, Okuda K, Kawase T, Hata K, Wolff LF, Yoshie H (2008) Tissue-engineered cultured periosteum used with platelet-rich plasma and hydroxyapatite in treating human osseous defects. J Periodontol 79(5):811–818
Yoshimura H, Muneta T, Nimura A, Yokoyama A, Koga H, Sekiya I (2007) Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. Cell Tissue Res 327(3):449–462
Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K et al (2001) Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec 264(1):51–62
Zimmermann T, Kunisch E, Pfeiffer R, Hirth A, Stahl HD, Sack U et al (2001) Isolation and characterization of rheumatoid arthritis synovial fibroblasts from primary culture–primary culture cells markedly differ from fourth-passage cells. Arthritis Res 3(1):72–76
Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ et al (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7(2):211–228
Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H et al (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13(12):4279–4295

Part III
Tissue Engineering, Biomaterials and Nanotechnology

Chapter 18
When Stemness Meets Engineering: Towards "Niche" Control of Stem Cell Functions for Enhanced Cardiovascular Regeneration

Maurizio Pesce, Giulio Pompilio, Gianluca Polvani, and Maurizio C. Capogrossi

Abstract The generation of bioartificial tissues using patient-derived or allogenic cells, has become a clinically relevant opportunity for translation in various branches of medicine, e.g. dermatology, ophthalmology and diabetes care. By contrast, despite the huge number of patients with cardiovascular diseases and the high economic burden, no feasible options exist to produce biomimetic engineered tissues that could be employed as definitive substitutes in cardiovascular medicine. In fact, while stem cells with cardiovascular competence have been identified and characterized, their employment has remained mainly confined to regenerative medicine, with insufficient translation into effective tissue engineering strategies. As a result, the devices presently available to replace diseased myocardium, occluded vessels and failing valves is limited to materials with tensile resistance (patches for ventricular reconstruction), autologous vessels (mammary/radial arteries and saphenous vein for aorto-coronary bypass grafts) and mechanical/bio-prosthetic valves, all of which have major limitations such as insufficient mechanical integration, post-engraftment patency reduction and calcification, respectively. Merging stem cell biology with recent bio-engineering techniques will be of great help in the production of new bio-synthetic cardiovascular medical devices. In fact, the ability to design complex biomaterial patterning in microscale or nanoscale dimensions and the

M. Pesce, Ph.D. (✉)
Laboratorio di Ingegneria Tissutale Cardiovascolare, Centro Cardiologico Monzino, IRCCS, Via C. Parea 4, I-20138 Milan, Italy
e-mail: maurizio.pesce@ccfm.it

G. Pompilio, M.D., Ph.D. • G. Polvani, Ph.D.
Dipartimento di Scienze Cardiovascolari, Università degli Studi di Milano, Via Parea, 4, 20138 Milan, Italy

M.C. Capogrossi, M.D.
Laboratorio di Patologia Vascolare, Istituto Dermopatico dell' Immacolata, IDI-IRCCS, Via dei Monti di Creta 104, I-00167 Rome, Italy

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_18,

ability to perform material-cell interaction analysis with a "high throughput" discovery power, can be exploited to obtain stem cell structuring in a similar fashion to natural "niche" conditions. In this way, the unique ability of stem cells to divide "asymmetrically" may be preserved, thus ensuring, at the same time, maintenance of an immature cell pool while enabling constant production of committed progenitors necessary for cellular renewal and tissue homeostasis.

18.1 Introduction. The Current Technical Limitations of Cardiovascular Regenerative Medicine

The ability to produce differentiated progenies maintaining tissue homeostasis and undifferentiated cellular pools, is a unique feature of stem cells that makes them an ideal resource for cell replacement strategies in organs with major insults. Transplanted cells, regardless of whether or not they strictly adhere to "stem" cell definitions, are expected to engraft into ischemic tissues, proliferate and differentiate into myocytes or vascular cells. Outstanding results obtained in animal models of human cardiovascular disease have shown that adult-derived stem cells (e.g. bone marrow-derived cells), contribute to myocardial regeneration through direct differentiation into cardiac myocyte-like derivatives (Orlic et al. 2001). However, it has been suggested that stem cell engraftment and cardiac differentiation have low efficiency and that cell therapy acts mainly *via* paracrine effects (Gnecchi et al. 2008). Despite encouraging results obtained in animal models, the real efficacy of cell therapy clinical translation is still controversial. In fact, meta-analysis studies have highlighted that efficacy of autologous-derived progenitors on recovery of heart function (i.e. ejection fraction, end diastolic/systolic volumes), remains limited (Abdel-Latif et al. 2007; Kang et al. 2008; Lipinski et al. 2007; Martin-Rendon et al. 2008a, b). The limited repair efficiency in the clinical setting may depend on inadequate progenitor cells preparation and/or delivery procedures (Seeger et al. 2007a, b), on poor progenitor cell survival in the recipient environment, or on an intrinsic reduction of their regeneration/repair ability, due to patients age and/or combined cardiovascular risk factors (Pesce et al. 2011a).

While significant advancements have been made toward the development of clinically approved stem cell preparation protocols with the introduction of "good manufacturing practice" (GMP) criteria (Dellatore et al. 2008; Gaipa et al. 2010), or the use of "cell enhancement strategies" to recover innate functions (Seeger et al. 2007b), "bulk" expansion using conventional methods appears insufficient to maintain "stemness" characteristics. In fact, exposure of stem cells to soluble cytokines or adhesion onto plastic-made rigid surfaces with stiffness of several orders or magnitude higher than tissue environment, does not preserve the innate ability of stem cells to asymmetrically divide and self renew (Dellatore et al. 2008) and may even promote aberrant differentiation. For example, expansion of high throughput flow sorted c-kit$^+$ CSCs from the human heart, even in the presence of supporting cells from the heart itself, did not prevent loss of stem cell properties and downregulation

of c-kit marker even after few passages (Gambini et al. 2011), while appropriate maintenance of stem cell self renewal ability in human cord blood derived $CD34^+$ cells culture was only in part ensured by use of epigenetic active drugs balancing the potent differentiation induction by cytokines supplemented in the culture medium to expand these cells (Burba et al. 2011).

18.2 What Is the "Necessity" for a Niche in Adult Tissues Organization?

Ever since the concept was proposed by Shofield in 1978 for describing the environment where primitive hematopoietic stem cells are allocated in the bone marrow, the term "niche" has been a source of confusion, controversy and intrigue. In fact, confinement of stem cells into special "enclaves", where they are protected from inductive signals coming from surrounding differentiated cells, may have been under-appreciated.

But what is a stem cell niche? This "home place" for stem cells (Jones and Wagers 2008) is, historically, very familiar to developmental biologists for description of the transient locations where primitive stem cells primary differentiation (or escape from differentiation) occurs during ontogenesis. A striking example is represented by the mammalian germline, which is specified by allocation of totipotent cells groups in the extra-embryonic mesoderm at specific stages of gastrulation to protect them from primary differentiation events (Pesce et al. 1998). Several niches contributing to cardiovascular differentiation of pluripotent cells have been recognized during vertebrate, mammalian and human embryogenesis and adulthood. For example, (**1**) the para-aortic mesenchyme, whence primitive hematopoietic cells emerge from an "hemogenic endothelium" through a novel type of cell division, named Endothelial-Hematopoietic transition (EHT) (Boisset et al. 2010; Kissa and Herbomel 2010; Lancrin et al. 2009; Tavian et al. 1999), or (**2**) the location where "clonal" expansion of multipotent progenitors occurs in the cardiac mesoderm, preceding specification of the two heart fields (Meilhac et al. 2004). From these basic examples it appears that niche "topology" is essential to sustain appropriate specification of stem cells, fulfilling their tissue organization role and functional development, in a tight equilibrium with the surrounding environment.

The function of the niches in adult tissues is different from that reported in the embryos. In fact, in the absence of tissue damage, the niche enables the replacement of differentiated cells, lost as a consequence of normal cellular turnover. This ensures tissue integrity and function over the life time. Under these conditions, the principal regulatory role of the niche is to maintain stem cells "asymmetric" division, a stem cell-specific cell division modality which maintains constant the number of primitive cells, while producing sufficient numbers of rapidly expanding progenitors necessary for tissue cellular turnover (Gonczy 2008). In case of tissue damage, or during pathologic progression, the niche is able to switch to a symmetric division modality. This change is necessary to produce high numbers of progenitors contributing

to tissue healing and repair. Reversibility between symmetric and asymmetric division, is a general and crucial mechanism ensuring a rapid response to damages and return to steady conditions. In fact, unnecessary overgrowth of undifferentiated cells after the end of the repair process may lead to immature cells accumulation (a condition often observed in cancer), while an excess of progenitors production in response to damage may cause stem cell exhaustion and premature tissue senescence (Tajbakhsh et al. 2009). From these concepts it is clear why stem cells and their niches have been recently described as "dynamic duos" (Voog and Jones 2010), whose components cannot be really taken apart. In fact, stem cells could not survive with a full potency, if it were not for their niches where their division modalities are finely tuned, while the niches themselves would be irreversibly fated to disappearance without the presence of stem cells.

18.3 A Bioengineering Conception of the Cardiovascular Niche. Is It Possible?

A major question arising in modern stem cell science is whether the niche environment is "computable" using quantitative methods, and whether this modeling activity may be eventually turned into experimental models, to be used for expanding stem cells with niche-like modalities (Dellatore et al. 2008; Kirouac and Zandstra 2008; Peerani et al. 2009). The adoption of a bioengineering vision of the stem cell niche might help overcome the current limitations imposed by stem cells "bulk" expansion resulting from translation of routinely used cell amplification procedures, and spark the devise of enhanced protocols to "fabricate" artificial niches (Becerra et al. 2011). These may be then used as "functional units" for populating cell-free artificial or natural scaffolds for the large scale production of biosynthetic tissues. This issue is particularly relevant for the recent devise of methodologies for decellularizing entire organs such as the liver, the lung and the heart, and seeding-back cultured cells into the resulting scaffolds (Ott et al. 2008, 2010; Uygun et al. 2010).

Considering its complexity, this endpoint is no simple matter for biologists alone. In fact, in addition to performing basic cell biology studies to identify extracellular signals, (epi)genetic factors and biochemical cues linked to basic stem cell differentiation and self renewal, other crucial components in this approach are: (**1**) the ability to manipulate biomaterials using combinatorial chemistry (Anderson et al. 2004; Tourniaire et al. 2006); (**2**) to reproduce compliant matrix stiffness and natural biomechanical forces/geometric constraints (Brown and Discher 2009; Discher et al. 2009); (**3**) to perform bio-computing to identify principal "nodes" in gene regulatory networks involved in cellular phenotype (Kirouac et al. 2009, 2010) and, finally, (**4**) to have access to "micro-fabrication" techniques to culture stem cells in complex microenvironments (microwells, micropatterned surfaces) (Kilian et al. 2010; Kurth et al. 2011; Wan et al. 2010, 2011), where to analyze cell phenotype with high throughput discovery potential.

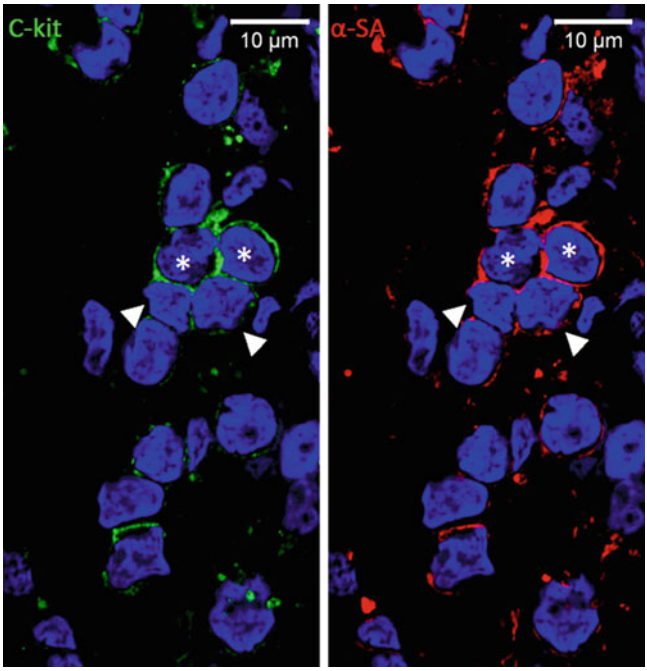


Fig. 18.1 Confocal microscopy high power view of a stem cell cluster located in the right atrium auricle appendage (Gambini et al. 2011). The picture shows staining for stem cell marker c-kit (*green fluorescence*) and alpha-Sarcomeric Actin (α-SA, *red fluorescence*). *Asterisks* show two cells expressing c-kit at high levels, in concert with α-SA, while *arrowheads* indicate cells expressing lower c-kit levels and α-SA. These cells represent cardiac-committed progenitors

18.3.1 Cardiovascular Niches

Like almost every specialized tissue in multicellular organisms, blood vessels, myocardium and heart valves undergo cellular turnover and repair, ensured by a coordinated activity of endothelial (Asahara et al. 1997), vascular- (Campagnolo et al. 2010; Crisan et al. 2008), myocardial- (Bearzi et al. 2007; Beltrami et al. 2003) and valve-resident (Taylor et al. 2003) stem cells. While all these stem cell types have been extensively characterized for their molecular characteristics and repair potency in experimental models and clinical trials, the intrinsic regulation of their homeostatic function has not been clarified.

Figures show, respectively, the structure of the supposed myocardial (Fig. 18.1) and vascular niches (Fig. 18.2), and the distribution of the cells inside the aortic valve leaflets (Fig. 18.3). From these images it is apparent that despite these structures contain cells with high degree of immaturity, they are structured and regulated in different fashions. In fact, they have various three-dimensional organization, different tissue homeostatic functions and are likely under the control of different biomolecular signalling. From a structural point of view, for example, the myocardial

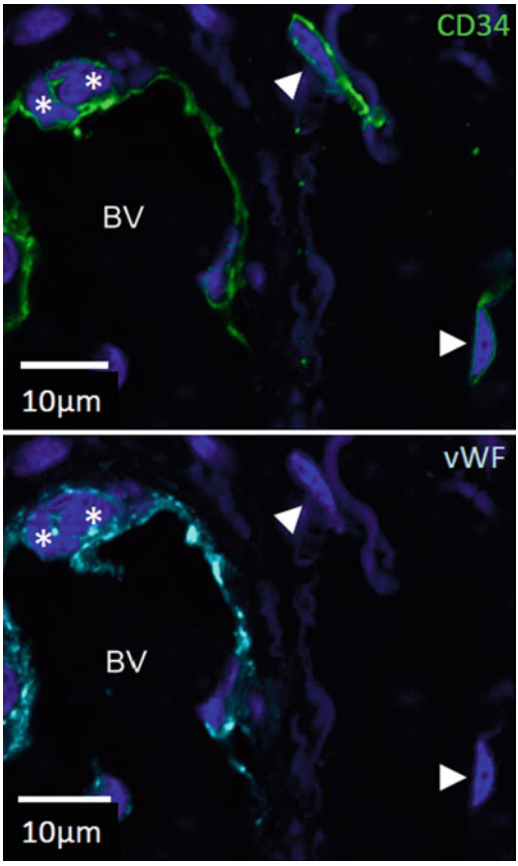


Fig. 18.2 Confocal microscopy image showing staining of a vessel present in the adventitia layer of human saphenous vein. *Green fluorescence* shows the expression of CD34, a marker expressed in endothelial cells and SVPs (Campagnolo et al. 2010), while *light blue fluorescence* indicates expression of von Willenbrand Factor, expressed in endothelial cells, but not SVPs. *Asterisks* indicate endothelial cells lining the lumen of the blood vessel (BV), *arrowheads* the location of $CD34^{+}/vWF^{-}$ SVPs surrounding the vessel

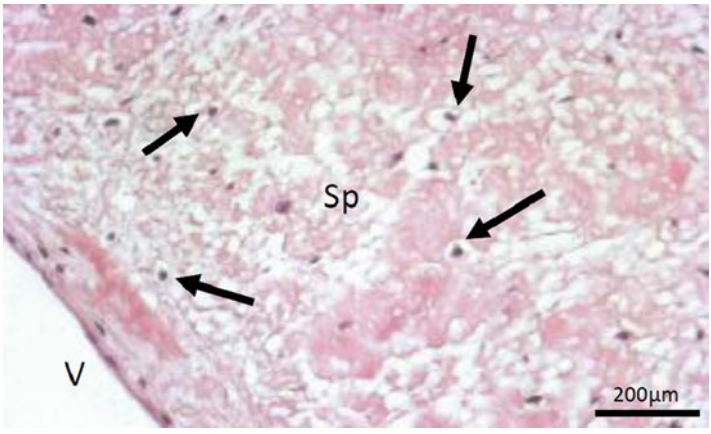


Fig. 18.3 Histology image of an aortic valve leaflet transversally cut. The presence of VICs (*arrows*) is evident in the intermediate layer of the leaflet, the so called *spongiosa* (Sp). The layer of the leaflet facing the ventricle cavity is shown (V)

niche is described spherical in shape, and composed of clusters of multipotent cells expressing the c-kit antigen (CD117) in the core, surrounded by accessory cells, likely originating from stem cells asymmetric own divisions (Gambini et al. 2011; Leri et al. 2005). By contrast, the vascular-resident niche has more of a ring-shaped structure containing pericyte-like mesenchymal stem cells surrounding the so called *vasa vasorum* (Majesky et al. 2011), located in the *adventitia* layer of large vessels, e.g. the saphenous vein (Campagnolo et al. 2010; Katare et al. 2011). Finally, in the aortic valves, the so called "valve interstitial cells" (VICs) closely resemble mesenchymal-like progenitors, are mostly localised in the inner leaflets layer called *spongiosa*, and do not appear to follow a specific pattern. This is likely due to the need for continuous extracellular matrix remodelling by these cells, which maintain correct leaflet stiffness and mechanical resistance (Chen et al. 2009). The structural and chemical heterogeneity of the cardiovascular niches makes their artificial conception and design an outstanding problem. In fact, these microenvironments not only differ for the geometric arrangement, but also for the chemical composition, the nature of supporting cells, and biochemical/biophysical extrinsic factors.

18.3.2 The Role of Mechanical Stress and Geometric Constraints in Phenotypic Control of Stem Cell Fate

Important components regulating the cardiovascular niche homeostasis are expected to be tissue-specific physical/chemical conditions. In fact, confinement of stem cells into environments with variable stiffness and mechanical strain, or with finely regulated geometries have been associated with activation of intracellular pathways "forcing" stem cells differentiation programs (Brown and Discher 2009; Discher et al. 2009; Mohyeldin et al. 2010).

18.3.2.1 Geometric Cues That Govern (Stem) Cell Programming

A striking example of finely tuned correlation between cellular phenotype and substrate mechanical compliance has been provided in a study where the expression level of gene sets related to neurogenic, myogenic and osteogenic phenotypes were finely controlled by culturing human-derived mesenchymal stem cells (MSCs) onto poly-acrylamide gels casted into culture wells with variable stiffness (Engler et al. 2006). Interestingly, progressive increase in the expression of osteogenic *vs.* neurogenic markers paralleled discrete increases in the gel "elastic modulus", as revealed by atomic force microscopy (AFM), while intermediate substrate stiffness corresponded to upregulation of myogenic markers and under-expression of the other two markers sets. In addition, plating the cells in the presence of media containing growth factors inducing MSCs differentiation failed to override the stiffness-related commitment, thus suggesting that environmental mechanical sensing is more potent than treatment with commonly used growth factors to direct MSCs differentiation (Engler et al. 2006). Other examples of controlled cell stimulation systems for

assessment of stem cells mechanobiology, were the recent creation of stiffness gradients by use of photo-polymerizable polyacrylamide gels placed onto glass slides over differential illumination by ultraviolet (UV) light (Tse and Engler 2011) or the functionalization of stretching membranes present in commercially available systems (e.g. Flex Cell™) with gels of controlled, tunable, stiffness (Throm Quinlan et al. 2011). Using these systems it was possible to assess the durotaxis ability (ability to migrate toward areas of stiffer matrix) of mesenchymal cells and to decipher phenotypic changes of mesenchymal stem cells and aortic VICs under dynamic strain. Importantly, from these reports, it emerged that migratory behavior, differential commitment, as well as cell shape and alignment to stretch direction are finely tuned by stiffness mechanosensing, likely involving transmembrane receptors such as integrins, connected to the surrounding matrix, and components of the cytoskeleton such as stress fibers.

What is the biological relevance of these findings? Recent work has suggested that biomechanics is in striking relationship with normally occurring pathophysiologic processes in the cardiovascular system. For example, it was shown that substrate stiffness is inversely correlated to expression of osteogenic differentiation markers in aortic VICs, even if calcium deposition by these cells was higher on stiffer substrates, due to enhanced VIC apoptosis-related calcification and stiffness-dependent responsiveness to TGF-β1, a potent inducer of calcification abundantly expressed in pathologic valve leaflets (Yip et al. 2009). This identifies changes in matrix elasticity occurring in the AoV inner layer, as a potent *primus movens* of valve calcification by promoting durotaxis and calcium deposition by locally recruited VICs, and suggests that VIC-mediated engineering of artificial valve leaflets will have to be performed with a tight control of material stiffness, to prevent accelerated calcification. Whether VICs are permanently modified at an (epi) genetic level by differential exposure to mechanic stimuli and local modification of the extracellular matrix, is still matter for speculation, and is currently addressed in our Laboratory. An example that clarifies how stem cells differentiation may influence the biomechanics of recipient tissues with consequences for tissue remodeling and function is relative to the change in mechanical compliance of ischemic areas, receiving injection of mesenchymal cells in the left ventricle (LV) following myocardial infarction (MI). In a study performed using AFM to quantify elastic modulus of the myocardial wall following MI, Berry et al. found an increasing stiffness likely due to collagen deposition (Berry et al. 2006). Interestingly, MSCs administration in the border zone of the infarct areas improved mechanical compliance (decrease in elastic modulus), possibly by attenuating infiltration by myofibroblasts, the cells responsible for collagen-I deposition and myocardial scarring following MI (Frangogiannis 2008). This suggests that an important outcome, even in the clinical setting, of (stem) cell injection in the ischemic heart, may be to attenuate the increase in myocardial compliance; this may account for the improvement in cardiac performance even in the absence of differentiation of the injected cells into myocytes. Whether and how exogenous stem cells injected into the infarcted myocardium cross-talk with the innate immunity pathways leading to myocardial scarring is still debated. On the other hand, as shown in a recent

contribution by our Laboratory (Burba et al. 2011), injection of stem cells can modify the interplay between different immunomodulatory cellular components, resulting into reduced recruitment of myofibroblasts, the cells involved in collagen deposition in the scarred myocardium (Carlson et al. 2011).

Elastomeric forces and geometric cues play important roles in cell programming and differentiation, with consequences for adoption of physiologic versus pathologic phenotypes. Several examples have been provided in the recent literature, which have made use of bioengineering approaches to dissect the impact of the forces generated by individual cells and to correlate them with the phenotype. In a first application, the use of elastomeric micro-needles allowed the measurement of cytoskeletal traction forces between adjacent cells as a function of the size of the so called "adherent" junctions (Liu et al. 2010). Interestingly, pharmacologic modulation of the force generated by cytoskeleton "engines", such as acto-myosin filaments regulated the size of the junctions, thus suggesting an environmental control of intercellular communication, and potentially, cell fate. A second example of a tight correlation between mechanical sensing and cell fate is witnessed by a series of studies, where cells were forced to stay in defined shapes by adhesion to geometrically defined surfaces built by the use of micro-contact printing (μCP) (Qian and Wang 2010), a gold standard technology for producing micro-shaped adhesion patterns. With this approach, it was found that MSCs have a preferential commitment into osteogenic cells when they are plated onto structures (i.e. star and wide rectangle shapes) forcing them to expose more extended perimeters; by contrast they had an adipogenic fate when plated onto surfaces maintaining them in less extended shapes, such as round or square adhesive patterns (McBeath et al. 2004; Peng et al. 2011).

How do cells sense geometry? Cell biology studies have highlighted that tension transmitted through cytoskeletal components such as acto-myosin (stress) fibers is likely implicated in geometric sensing through the differential activity of the RhoA small GTPase and its downstream target RhoA-associated kinase ROCK (Bhadriraju et al. 2007). Interestingly, ROCK activity was elevated by a more spread shape, priming cells into osteogenic lineage, while round or loosely attached cell configurations were associated to higher RhoA-GTP level, lower ROCK activity and adipogenic phenotype (McBeath et al. 2004). In a recent study, the concept of geometric priming of MSCs adipogenic *vs.* osteogenic phenotypes has been further elucidated. By comparing cells which were forced to acquire a "flower" (less spread) *vs.* a "star" (more spread) shape, it was found that cells with more spread phenotype showed higher expression of genes encoding for mechanosensing, actomyosin contractility and receptors for canonical/non canonical Wnt pathway, in addition to genes directly implicated in osteogenesis (Kilian et al. 2010). Again, the cytoskeleton integrity, and in particular the actomyosin fibers and ROCK downstream activity, were crucial in this; in fact, disruption of these filaments by Cytochalasin-D (an inhibitor of F-actin polymerization), blebbistatin (an inhibitor of the light myosin contractility), or the use of Y-27632 (a ROCK inhibitor), reduced MSCs osteogenic commitment irrespective of cell shape and differentiation treatment. The involvement of small GTPases on shape-induced MSCs commitment appears of more general importance than for the sole

adipogenic *vs.* osteogenic differentiation. In fact, in another recent study, it was found that Rac-1, another RhoA small GTPase family member, has a role in TGF-β3-mediated commitment of spread MSCs into smooth muscle cells, and it is sufficient to repress chondrogenic commitment (Gao et al. 2010). Importantly, in this report, it was shown that upregulation of Rac-1 was also related to elevation of N-Cadherin expression, thereby highlighting the role of intercellular communication for smooth muscle cells commitment.

Cell differentiation is likely not the unique cell response modulated by shape. In fact, using a combination of μCP technology and electric field-assisted cell entrapment (dielectrophoresis), it was possible to place cells in discrete shapes and regulate the amount of intercellular communications (Gray et al. 2008). By this approach it was found that endothelial cells have a biphasic DNA synthesis pattern, which depends on the amount of the cell surface occupied by contacts with other cells and that engagement of intercellular adhesion molecule VE-Cadherin, rather than stress fibers tension modulated by RhoA, is crucial to activate a proliferation program.

Taken together, these results suggest that geometric arrangement, dictated by extracellular environment architecture and composition is a potent morphogenetic cue involved in various cell commitment programs during morphogenesis. Future bioengineering modeling of cell shape will be needed to address the role of these cues for (epi)genetic control of cell-autonomous cardiovascular stem cells development and pathologic differentiation.

18.3.2.2 Application of Geometric Constraints to Stem Cell Colonies: A Way to Simulate and Stimulate the Niche?

The application of μCP technology to confine cells in defined shapes has not been only used to assess the response of individual cells to physical constraints. In fact, "micro-patterned" bi-dimensional (2D) or, even three-dimensional (3D) structures have be adapted to culture stem cells colonies into environments that can be of help to study the "topology" of stem cell dynamics.

A first example of the application of this strategy to niche-like modelling of stem cells growth was described in a report from CM Nelson and colleagues (Nelson et al. 2005), showing the use of micro-patterned culture surfaces to study endothelial cell proliferation. By measuring the level of BrdU uptake in cells adherent to different geometric patterns (square, rectangle, annulus) by conventional immunofluorescence followed by data digitalization, the Authors were able to create "heatmaps" describing the geometric distribution of the cellular proliferation probability into the micro-pattern. Interestingly, they found that at the edges of the patterns, where mechanical stress was predicted to be higher, cell proliferation was more intense, while at the centre it was null of absent. By inhibiting the function of proteins involved in mechanical sensing, they were able to dysregulate cell proliferation inside the colony, thus showing an important relationship between mechanosensing and modulation of cellular proliferation.

A second example is the recent finding that (stem) cells have a lineage-dictated ability to orient their major axes with a preferential angle when plated onto micropatterns. By fabricating annulus-shaped surfaces for culturing these cells, and using a software platform making possible automatic cell shape recognition and orientation in the 2D space, it was shown that different cell lines have a recurrent positioning of their major axis relative to the micropattern radius, which is dictated by their phenotype (Wan et al. 2011). The probability of creating patterns with cells oriented in clockwise (CW) *vs.* counter clockwise (CCW) directions relative to the pattern radius reached, in some cell lines, exhibited extremely high significance ($P = 10^{-186}$), showing that the alignment preference of the cells was not stochastic. Furthermore, the lineage-specific orientation was conserved among species. In fact mouse and human-derived myoblasts showed CCW orientation, while mouse, human and rat-derived fibroblasts acquired a CW orientation. This suggests an (epi)genetic commitment of cellular left-right asymmetry (chirality), which may be involved in fundamental morphogenetic events, such as organ looping, or important regulatory components of stem cell asymmetric division.

As described for single cells cultured onto micropatterns, geometric cues also direct stem cell spatial commitment when plated into colonies of defined shapes. For example, by placing scalable amounts of MSCs onto circular micro-patterns of increasing radius, it was found that osteogenic and adipogenic commitments were spatially organized depending on the position of the cells in the micropattern, while the ratio between osteogenesis and adipogenesis was correlated to the dimension of the cellular aggregates (Ruiz and Chen 2008; Wan et al. 2010). Interestingly, in all patterns that were used, adipogenesis occurred principally in the centre of round-shaped, or at the concave surface of offset annulus or sinusoidal-shaped colonies, indicating that topology of the cellular aggregates, probably related to gradients of mechanical stress (Nelson et al. 2005), is crucial to direct selective commitment of progenitors endowed with multilineage potential.

These results suggest that the ability to discriminate between different geometric arrangements is an intrinsic regulatory feature of stem cells inside the niche. Thus, the ability to quantify cellular responses to colony geometries, might be a powerful method for quantifying the niche by artificial trapping stem cells into defined micropatterns and screening changes in (epi)genetic signatures associated to, e.g. symmetric *vs.* asymmetric division modality, or to differentiation. This computing ability is today possible by use of automated high content microscopy, programmable to obtain quantitative phenotypic analysis of cells confined into microenvironments, and high-end translation of data generated by cell-cell/cell-substrate interactions into microarray-like numeric data, enabling to perform multiple comparisons, clusterization, and network-level analysis of intercellular cross-talk by suitable bioinformatics platforms. As already performed in pioneering investigations performed using hematopoietic stem cells or embryonic stem cells in geometric-independent environments (Kirouac et al. 2009, 2010; Peerani et al. 2009), these approaches will be crucial to make future *in silico* predictions of stem cell dynamics inside the natural niches, and to reproduce them artificially (Lutolf et al. 2009).

18.4 Reproducing the "Combinatorial Chemistry" of the Stem Cell Niche with High Throughput Discovery Power

The maintenance of appropriate functional appearance of stem cells in the niche, the so-called "functiotype", is the result of *"complex interactions integrating molecular, extracellular, biomechanical and spatio-dimensional effects"* (Roeder et al. 2011), whose microscale/nanoscale arrangements are necessary for instructing coherent stem cells differentiation, or for reversibly shifting niches from "dormant" to "active" stages in case of tissue damage. The chemistry of the microenvironment, in addition to spatial cues, is particularly relevant for this reversible dynamics. In fact, multiple paracrine signals expressed in normal tissues or up/down-modulated as a result of tissue damage or pathology may control stem cells dynamics in living conditions. This regulation may also involve other factors such as, for example, oxygen tissue availability or extracellular matrix composition. It is beyond the scope of the present article to describe in details these pathways, already treated by us and by others in various contributions (Keung et al. 2010; Mohyeldin et al. 2010; Pesce et al. 2011a, b). On the other hand, experimental modelling of the complex niche chemistry appears crucial to correctly reproduce stem cells functiotype in the niche.

Recent refinement in biocompatible polymer generation and screening have opened new avenues in the screening of cell/material interactions. These systems are based on "combinatorial" organic chemistry, and exploit an "inkjet" printing-derived technology to create microarrays of up to 7,000 micrometric spots containing different polymeric blends onto a single glass slide. While providing a miniaturization of the cell culture areas, these systems, similar to μCP, allow a rapid and efficient identification of culture substrates maximizing cell attachment, proliferation, spreading and, potentially several other responses. As in the case of stem cells colonies geometric modelling onto micropatterns (Nelson et al. 2005), screening of cellular behaviour in response to polymeric blends is performed by automated high content microscopy, which allows interrogation of cells cultured onto the microarrays, followed by computer-assisted results digitalization. By this approach, various libraries of materials such as acrylate-based polymers (Anderson et al. 2004), polyurethanes (Tourniaire et al. 2006) and synthetic/natural hydrogels mixtures (Khan et al. 2009; Zhang et al. 2009) have been screened to identify the best biomaterials promoting adhesion and proliferation of various (stem) cell types such as embryonic stem cells (Anderson et al. 2004), fibroblasts (Pernagallo et al. 2008) and hepatocytes (Hay et al. 2011).

The versatility offered by the "high throughput" design of this polymer screening system offers a tremendous potential in terms of stem cell niche modelling. In fact, materials to be used for growing stem cells can be screened directly "on array" for several characteristics such as surface roughness, wettability or elastic modulus. Data obtained from cells interrogations onto these materials may be then converted into "association maps" which describe complex cell behaviour in response to multiple material characteristics (Mei et al. 2010). In addition, the possibility of performing association between material composition, as detected by analytical

chemistry methods, such as Time-Of-Flight/Secondary-Ion-Mass-Spectrometry (TOF-SIMS), and cellular responses by Partial Least-Square (PLS) chemometric method, makes possible to perform statistically validated predictions of the cell behaviour in response to material chemistry, and thus design stem cell type-tailored materials by *in silico* simulation (Davies et al. 2010).

Another advantage offered by material screening with a high throughput experimental design is related to the variety of biological functionalization of synthetic materials, which may be produced onto single arrays and screened in the same experiments, with the aim of refining the chemistry in stem cells cultured colonies. In fact, starting from the natural composition and the geometric arrangement of tissues-specific niches, such as in the myocardium, the vessels and the heart valves, *bio*-functionalization of the synthetic materials might be performed by covalent binding of extracellular matrix components, peptides mimicking matrix binding motifs, or even niche specific growth factors involved in stem cell regulation in given geometric arrangements (Dellatore et al. 2008; von der Mark et al. 2010). In this way, secondary *bio*-functionalized material arrays might be generated and again interrogated for stem cell regulation, thus identifying refined chemistry conditions for instructing niche auto assembly in defined geometric arrangements. Definition and release of these "smart" materials might lead, finally, to the generation of novel approaches, based on the natural ability of niches to (re)populate diseased tissues or natural decellularized/artificial scaffolds for the generation of novel implant devices such as artificial myocardium patches, biologically compatible valve leaflets or engineered vessels.

18.5 A Glimpse to the Future

The field of tissue engineering has taken great strides in the previous two decades, so as to develop biological solutions to repair damaged organs and tissues within the body. For example, the recent development of protocols to remove endogenous cells and re-seed, using *ex vivo* cultured cells, organs such as the liver, the lung and the heart (Ott et al. 2008, 2010; Uygun et al. 2010), have provided proof of principle that generation of living and fully functioning artificial organs might be realistic. In our view, a major limit in the generation of bioartificial organs is the lack of a sufficient vision to the need for generating organs with self-renewing characteristics. In fact, the use of differentiated cells (e.g. cardiomyocytes, endothelial cells, smooth muscle cells) may have the advantage to exploit already available "bulk" expansion protocols, which can be easily adapted to comply with good manufacturing practice (GMP) standards, required for clinical use of stem cells (Burba et al. 2010). On the other hand, the failure to include self-renewing stem cells in artificial or decellularized natural scaffolds might lead to rapid failure of bioartificial implants due to cellular exhaustion.

As discussed in the present contribution, the rapid development of miniaturized devices, the so called lab-on-chips, to perform cell biology experiments with high

throughput discovery potential (Ghafar-Zadeh et al. 2011) will provide growing teams of basic biologists, biotechnologists, chemists, engineers and bio-computing scientists with extremely powerful methods to assess global changes in stem cells regulation in a relatively unexpensive manner. It is possible that these methods, helped by novel technologies to produce micro-encapsulated cells to be "printed" into tissues (Hernandez et al. 2010; Kachouie et al. 2010), are eventually converted into procedures for generating next generation implants, having structures and functions more closely related to those in their target organs.

References

Abdel-Latif A et al (2007) Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. Arch Intern Med 167:989–997

Anderson DG et al (2004) Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. Nat Biotechnol 22:863–866

Asahara T et al (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967

Bearzi C et al (2007) Human cardiac stem cells. Proc Natl Acad Sci U S A 104:14068–14073

Becerra J et al (2011) The stem cell niche should be a key issue for cell therapy in regenerative medicine. Stem Cell Rev Rep 7(2):248–255, Humana Press Inc

Beltrami AP et al (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114:763–776

Berry MF et al (2006) Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. Am J Physiol Heart Circ Physiol 290:H2196–H2203

Bhadriraju K et al (2007) Activation of ROCK by RhoA is regulated by cell adhesion, shape, and cytoskeletal tension. Exp Cell Res 313:3616–3623

Boisset JC et al (2010) In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature 464:116–120

Brown AEX, Discher DE (2009) Conformational changes and signaling in cell and matrix physics. Curr Biol 19:R781–R789

Burba I et al (2010) When cells become a drug. Endothelial progenitor cells for cardiovascular therapy: aims and reality. Recent Pat Cardiovasc Drug Discov 5:1–10

Burba I et al (2011) Histone deacetylase inhibition enhances self renewal and cardioprotection by human cord blood-derived CD34 cells. PLoS One 6:e22158

Campagnolo P et al (2010) Human adult vena saphena contains perivascular progenitor cells endowed with clonogenic and proangiogenic potential. Circulation 121:1735–1745

Carlson S et al (2011) Cardiac mesenchymal stem cells contribute to scar formation after myocardial infarction. Cardiovasc Res 91(1):99–107

Chen JH et al (2009) Identification and characterization of aortic valve mesenchymal progenitor cells with robust osteogenic calcification potential. Am J Pathol 174:1109–1119

Crisan M et al (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3:301–313

Davies MC et al (2010) High throughput surface characterization: a review of a new tool for screening prospective biomedical material arrays. J Drug Target 18:741–751

Dellatore SM et al (2008) Mimicking stem cell niches to increase stem cell expansion. Curr Opin Biotech 19:534–540

Discher DE et al (2009) Growth factors, matrices, and forces combine and control stem cells. Science 324:1673–1677

Engler AJ et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126:677–689

Frangogiannis NG (2008) The immune system and cardiac repair. Pharmacol Res 58:88–111
Gaipa G et al (2010) GMP-based CD133+ cells isolation maintains progenitor angiogenic properties and enhances standardization in cardiovascular cell therapy. J Cell Mol Med 14:1619–1634
Gambini E et al (2011) C-kit+ cardiac progenitors exhibit mesenchymal markers and preferential cardiovascular commitment. Cardiovasc Res 89:362–373
Gao L et al (2010) Stem cell shape regulates a chondrogenic versus myogenic fate through Rac1 and N-cadherin. Stem Cells 28:564–572
Ghafar-Zadeh E et al (2011) Engineered approaches to the stem cell microenvironment for cardiac tissue regeneration. Lab Chip 11(18):3031–3048
Gnecchi M et al (2008) Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 103:1204–1219
Gonczy P (2008) Mechanisms of asymmetric cell division: flies and worms pave the way. Nat Rev Mol Cell Biol 9:355–366
Gray DS et al (2008) Engineering amount of cell-cell contact demonstrates biphasic proliferative regulation through RhoA and the actin cytoskeleton. Exp Cell Res 314:2846–2854
Hay DC et al (2011) Unbiased screening of polymer libraries to define novel substrates for functional hepatocytes with inducible drug metabolism. Stem Cell Res 6:92–102
Hernandez RM et al (2010) Microcapsules and microcarriers for in situ cell delivery. Adv Drug Deliv Rev 62:711–730
Jones DL, Wagers AJ (2008) No place like home: anatomy and function of the stem cell niche. Nat Rev Mol Cell Biol 9:11–21
Kachouie NN et al (2010) Directed assembly of cell-laden hydrogels for engineering functional tissues. Organogenesis 6:234–244
Kang S et al (2008) Effects of intracoronary autologous bone marrow cells on left ventricular function in acute myocardial infarction: a systematic review and meta-analysis for randomized controlled trials. Coron Artery Dis 19:327–335
Katare R et al (2011) Transplantation of human pericyte progenitor cells improves the repair of infarcted heart through activation of an angiogenic program involving micro-RNA-132. Circ Res 109(8):894–906
Keung AJ et al (2010) Presentation counts: microenvironmental regulation of stem cells by biophysical and material cues. Annu Rev Cell Dev Biol 26:533–556
Khan F et al (2009) Versatile biocompatible polymer hydrogels: scaffolds for cell growth. Angew Chem Int Ed Engl 48:978–982
Kilian KA et al (2010) Geometric cues for directing the differentiation of mesenchymal stem cells. Proc Natl Acad Sci U S A 107:4872–4877
Kirouac DC, Zandstra PW (2008) The systematic production of cells for cell therapies. Cell Stem Cell 3:369–381
Kirouac DC et al (2009) Cell-cell interaction networks regulate blood stem and progenitor cell fate. Mol Syst Biol 5:293
Kirouac DC et al (2010) Dynamic interaction networks in a hierarchically organized tissue. Mol Syst Biol 6:417
Kissa K, Herbomel P (2010) Blood stem cells emerge from aortic endothelium by a novel type of cell transition. Nature 464:112–115
Kurth I et al (2011) Extracellular matrix functionalized microcavities to control hematopoietic stem and progenitor cell fate. Macromol Biosci 11:739–747
Lancrin C et al (2009) The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. Nature 457:892–895
Leri A et al (2005) Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev 85:1373–1416
Lipinski MJ et al (2007) Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials. J Am Coll Cardiol 50:1761–1767
Liu Z et al (2010) Mechanical tugging force regulates the size of cell junctions. Proc Natl Acad Sci U S A 107:9944–9949

Lutolf MP et al (2009) Designing materials to direct stem-cell fate. Nature 462:433–441
Majesky MW et al (2011) The adventitia. Arterioscler Thromb Vasc Biol 31:1530–1539
Martin-Rendon E et al (2008a) Stem cell treatment for acute myocardial infarction. Cochrane Database Syst Rev 2008(4):CD006536
Martin-Rendon E et al (2008b) Autologous bone marrow stem cells to treat acute myocardial infarction: a systematic review. Eur Heart J 29:1807–1818
McBeath R et al (2004) Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell 6:483–495
Mei Y et al (2010) Combinatorial development of biomaterials for clonal growth of human pluripotent stem cells. Nat Mater 9:768–778
Meilhac SM et al (2004) The clonal origin of myocardial cells in different regions of the embryonic mouse heart. Dev Cell 6:685–698
Mohyeldin A et al (2010) Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell 7:150–161
Nelson CM et al (2005) Emergent patterns of growth controlled by multicellular form and mechanics. Proc Natl Acad Sci U S A 102:11594–11599
Orlic D et al (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410:701–705
Ott HC et al (2008) Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med 14:213–221
Ott HC et al (2010) Regeneration and orthotopic transplantation of a bioartificial lung. Nat Med 16:927–933
Peerani R et al (2009) Manipulation of signaling thresholds in "engineered stem cell niches" identifies design criteria for pluripotent stem cell screens. PLoS One 4:e6438
Peng R et al (2011) Effect of cell anisotropy on differentiation of stem cells on micropatterned surfaces through the controlled single cell adhesion. Biomaterials 32:8048–8057
Pernagallo S et al (2008) Deciphering cellular morphology and biocompatibility using polymer microarrays. Biomed Mater 3:034112
Pesce M et al (1998) In line with our ancestors: Oct-4 and the mammalian germ. Bioessays 20:722–732
Pesce M et al (2011a) Endothelial and cardiac progenitors: boosting, conditioning and (re)programming for cardiovascular repair. Pharmacol Ther 129:50–61
Pesce M et al (2011b) Cardiac stem cells: tales, mysteries and promises in heart generation and regeneration. Regen Med. Springer Netherlands, pp 265–286
Qian T, Wang Y (2010) Micro/nano-fabrication technologies for cell biology. In: Medical and biological engineering and computing, vol 48. Springer, Berlin/Heidelberg, pp 1023–1032
Roeder I et al (2011) Towards a quantitative understanding of stem cell-niche interaction: experiments, models, and technologies. Blood Cells Mol Dis 46:308–317
Ruiz SA, Chen CS (2008) Emergence of patterned stem cell differentiation within multicellular structures. Stem Cells 26:2921–2927
Seeger FH et al (2007a) Cell isolation procedures matter: a comparison of different isolation protocols of bone marrow mononuclear cells used for cell therapy in patients with acute myocardial infarction. Eur Heart J 28:766–772
Seeger FH et al (2007b) Cell-enhancement strategies for the treatment of ischemic heart disease. Nat Clin Pract Cardiovasc Med 4(Suppl 1):S110–S113
Tajbakhsh S et al (2009) Asymmetric cell divisions and asymmetric cell fates. Annu Rev Cell Dev Biol 25:671–699
Tavian M et al (1999) Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo. Development 126:793–803
Taylor PM et al (2003) The cardiac valve interstitial cell. Int J Biochem Cell Biol 35:113–118
Throm Quinlan AM et al (2011) Combining dynamic stretch and tunable stiffness to probe cell mechanobiology in vitro. PLoS One 6:e23272 EP
Tourniaire G et al (2006) Polymer microarrays for cellular adhesion. Chem Commun (Camb) 28(20):2118–2120
Tse JR, Engler AJ (2011) Stiffness gradients mimicking *in vivo* tissue variation regulate mesenchymal stem cell fate. PLoS One 6:e15978 EP

Uygun BE et al (2010) Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat Med 16:814–820
von der Mark K et al (2010) Nanoscale engineering of biomimetic surfaces: cues from the extracellular matrix. Cell Tissue Res 339:131–153
Voog J, Jones DL (2010) Stem cells and the niche: a dynamic duo. Cell Stem Cell 6:103–115
Wan LQ et al (2010) Geometric control of human stem cell morphology and differentiation. Integr Biol 2:346–353
Wan LQ et al (2011) Micropatterned mammalian cells exhibit phenotype-specific left-right asymmetry. Proc Natl Acad Sci 108:12295–12300
Yip CY et al (2009) Calcification by valve interstitial cells is regulated by the stiffness of the extracellular matrix. Arterioscler Thromb Vasc Biol 29:936–942
Zhang R et al (2009) Microarrays of over 2000 hydrogels – identification of substrates for cellular trapping and thermally triggered release. Biomaterials 30:6193–6201

Chapter 19
Vector Technology and Cell Targeting: Peptide-Tagged Adenoviral Vectors as a Powerful Tool for Cell Specific Targeting

Julia Reetz, Ottmar Herchenröder, Anke Schmidt*, and Brigitte M. Pützer*

Abstract A pre-requisite for efficient and successful treatment of diseases consists in the development of technologies which yield the transfer of therapeutic genes and drugs exclusively to target cells and avoid therapy related effects or toxicity in normal cells. Targeted gene therapy is emerging as a powerful approach to enhance the efficacy, selectivity and safety of gene delivery. Currently, the most efficient and popular way of introducing genes into cells is by means of viral vectors. Attractive targeting strategies of viruses are either by regulation of transgene expression through tissue specific promoters and integration of transcriptionally active elements (molecular targeting), or by selective recognition of individual cellular receptors (physical or transductional targeting). The latter can be achieved by alteration of the native viral tropism in conjunction with redirection of binding to target cell receptors. Receptor-targeting will be performed by linking or integration of adapter molecules to the viral surface (genetic modification). On the basis of recent knowledge and different limitations in practice, our focus is concentrated on the improvement of adenoviral vector systems for selective transduction of different cell types by directing adenovirus (Ad) to specific ligands. Those peptide-tagged Ad vectors have shown impressive tumor and stem cell specific gene transfer activity in vitro and after systemic administration in several in vivo models.

---

* Authors contibuted equally.

J. Reetz • O. Herchenröder, Ph.D. • B.M. Pützer, M.D., Ph.D. (✉)
Department of Vectorology and Experimental Gene Therapy, Biomedical Research Center, Rostock University Medical Center, Schillingallee 69, 18057 Rostock, Germany
e-mail: brigitte.puetzer@med.uni-rostock.de

A. Schmidt, Ph.D. (✉)
Zentrum für Innovationskompetenz (ZIK) plasmatis, INP Greifswald e.V.
Felix-Hausdorff-Str. 2, 17489 Greifswald, Germany

Department of Vectorology and Experimental Gene Therapy,
Biomedical Research Center Rostock University Medical Center
Schillingallee 69, 18057 Rostock, Germany

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_19,

They not only offer new opportunities to further delineate stem cell properties in their natural environment but may also enable more effective cancer therapies for patients with metastatic disease.

19.1 Introduction: A Historical Perspective

19.1.1 Gene Therapy and Delivery Vehicles

Gene therapy is based on the delivery of genes containing information for correction of either monogenetic disorders or treatment of more complex diseases such as cancer. One of the major hurdles towards successful gene therapy of inherited or acquired diseases is the efficient and safe selective introduction of the desired gene into the cells of interest under in vivo conditions. In addition, in order to ameliorate genetic diseases, long-term expression of the transgene is another prerequisite. In contrast to inherited monogenetic diseases, where only a certain proportion of cells can be targeted with a vector carrying a single corrective gene, cancer treatment usually involves efficient transduction of all neoplastic cells with the ultimate aim of killing rather than correcting them. Progress in the identification of molecular and genetic defects and high throughput screening technologies that accelerated the discovery of cell specific surface markers have made gene therapy an attractive treatment option and led to the development of selective and effective vehicles for transferring genes into cells. As a basis for clinical applications, technical advances have been made with a huge number of non-viral or viral gene delivery systems. However, most delivery methods to physically introduce non-viral plasmid DNA vectors are not very efficient and result in short duration of transgene expression especially in vivo (Patil et al. 2005; Preuss and Curiel 2007; Witlox et al. 2007; Alvarez-Erviti et al. 2011). Biological transfer particles beyond viral vectors and the strategies, advantages and limitations of established delivery techniques for gene therapy applications have been reviewed in detail by several authors (Seow and Wood 2009; Guo and Huang 2012; Koynova and Tenchov 2011; Duan 2011; Jafari et al. 2012). In comparison to DNA transfer using bacteria, bacteriophages, virus-like particles or exosomes, genetically modified viral vectors serve as more powerful tools for targeted gene therapy. They include integrating retroviral (lentiviral) and adeno-associated viruses (AAV) as well as non-integrating lentiviral, herpes simplex virus (HSV) or adenoviral (Ad) vectors.

AAV based vectors are used for gene therapy (Ortolano et al. 2012) because of several advantages: they infect both, dividing and non-dividing cells, induce moderate immune-responses and integrate for stable and long-term expression of therapeutic drugs. Drawbacks of AAV vectors are their restricted packaging capacity and the helper virus requirement for vector production which can result in low titer and purity (Bjorklund et al. 2000; Zhang and Godbey 2006; Witlox et al. 2007). However, several clinical trials with AAV are on-going or in preparation, trying to treat the inherited deficiency in clotting factor VIII (Hu et al. 2011) or the eye disease so called Leber's congenital amaurosis (Simonelli et al. 2010; Stein et al. 2011). The latter is an inherited disease of the retina and was treated successfully by AAV type 2 virus

vectors (AAV2) carrying the RPE65 gene (Bennett et al. 2012). Other approaches have been initiated to deliver genes into the brain (Morgenstern et al. 2011). In recent trials, AAV infected cells were recognized as compromised by the immune system and were, accordingly, killed by CD8 positive cells (Mingozzi and High 2011). This reaction appears to be triggered in part by capsid or outer coat proteins of AAV2 vectors (Bartel et al. 2011). AAV serotypes can differ with respect to the receptors they recognize. AAV2 for instance, binds to heparan sulfate proteoglycan, $\alpha_V\beta_5$ integrin and fibroblast growth factor receptor 1 (Qiu et al. 2000; Pajusola et al. 2002).

Most herpes simplex virus based vectors are mainly derived from the neurotropic human HSV-1 and are broadly used for treatment of neurodegenerative diseases (Casper et al. 2002; Zhang and Godbey 2006). Two approaches have been applied to construct recombinant HSV vectors for use in gene transfer. One class of vectors is made deficient for accessory viral functions important for virus replication in neurons and thereby, contributing to neurovirulence such as the thymidine kinase gene (TK). Another class is rendered defective in essential virus genes and thus fails to replicate in all cell types (Glorioso and Fink 2009). The combination of AAV, Ad vectors and HSV-based systems allows large-scale vector production for clinical studies (Clement et al. 2009).

Permanent transgene expression and easy handling made retroviral vectors the first efficient and most popular gene transfer system for disease treatment. However, the oncovirus subfamily of retroviruses infects only dividing cells and they are associated with malignancy or immune defects (Coffin 2000; Geraerts et al. 2006). Moreover, the chief concerns about this approach are frequent transgene silencing in vivo and potential activation of oncogenes after nearby virus integration (Vroemen et al. 2005). The lentivirus subfamily of retroviruses is a class of highly pathogenic viruses that are able to infect both, non-dividing and dividing cells. This feature has rendered human immunodeficiency virus (HIV) based gene delivery vehicles the frontrunners in the field. Expanding the HIV host range or specific targeting can be achieved by pseudotyping the lentiviral particles (Cronin et al. 2005; Schambach et al. 2006; Carpentier et al. 2012). These vectors show a long-term expression after chromosomal integration of large inserts, which makes them highly suitable for applications in the adult central nervous system (CNS) (Bjorklund et al. 2000; Witlox et al. 2007). Moreover, modifications of lentiviruses such as the replacement of long terminal repeats with cell specific transcription-regulatory sequences, promoters and enhancers have been made to allow an endothelial specific expression (Trono 2000; Shichinohe et al. 2001; Lotti et al. 2002; Dong and Nor 2009). Severe combined immunodeficiency (SCID) due to adenosine deaminase (ADA) deficiency was the first genetic disease treated by gene therapy. The obstacle with oncogene activation (Hacein-Bey-Abina et al. 2003) has recently been addressed by utilizing zinc finger nucleases or by including certain sequences such as the beta globin locus control region, to direct the site of integration towards specific chromosomal sites. Gene therapy trials in the USA to treat SCID were, however, interrupted after 3 out of 11 patients treated developed leukemia (Check 2002; Huston et al. 2011) while ten SCID patients treated in England did to date not present with leukemia and experienced sufficient immune recovery to enable a normal development (Baum 2011). No adverse leukemic side effects were seen using human leukocyte antigen-matched allogeneic hematopoietic stem cell transplantation as an enzyme replacement therapy for SCID (Gaspar et al. 2011).

Apart from the above described vectors, adenoviruses are attractive tools to construct gene delivery vehicles (Armendariz-Borunda et al. 2011). Wild-type adenoviruses are common pathogens in humans with a non-enveloped capsid containing a continuous double stranded DNA. All adenovirus serotypes display a broad tropism and infect a wide spectrum of cells (Arnberg 2009; Chailertvanitkul and Pouton 2010). Ad-based vectors grow to high titers and, after administration, do not integrate into the host genome. Most of the therapeutically used so-called first generation Ad vectors are replication-deficient after substitution of the E1 and E3 regions with foreign genes (Mizuguchi et al. 2001). Advanced third generation Ads brought considerable progress on issues like gene delivery capacity and long-term expression of the transgene (Viru et al. 2011) as well as low in vivo toxicity and immunogenicity. These high-capacity (HC) vectors also called gutless or helper-dependent (HD) Ad vectors lack all viral coding sequences resulting in a high packaging capacity of up to 36 kilobases. HD vectors allow the simultaneous expression of several therapeutic and/or marker genes (Dormond and Kamen 2011) and stable transgene expression. They have preferences over first generation vectors whenever long-lasting gene expression is required to correct genetic diseases (Brunetti-Pierri and Ng 2008, 2011). In a monogenic hyperlipidemia mouse model, a single injection of an HD-Ad vector expressing the missing protein resulted in lifelong protection of the animals from the respective condition (Kim et al. 2001). An efficient and scalable procedure for HC Ad vector production in a L3 bioreactor was developed using polyethylenimine-adenofection (Dormond et al. 2009; Dormond and Kamen 2011; Galvez et al. 2012). Adenovirus serotype 5 that has largely been used in clinical trials causes mild upper respiratory infections (Kajon and Erdman 2007; Kajon et al. 2007) but until now, no human neoplastic disease associates with Ad vectors. Altogether this suggests that different generations of Ad vectors will suit best compared to other viral or non-viral vector systems to transfer genes into target cells (Parks 2000; van Beusechem et al. 2002; Wickham 2003; Volpers and Kochanek 2004).

19.2 Background/Principles

19.2.1 Strategies for Targeting Adenoviral Vectors

The efficiency of adenovirus as gene delivery system (Coughlan et al. 2010) is limited by its native tropism that allows the virus to infect a broad range of cells and tissues, thereby preventing selective gene transfer. This property imposes an increased risk of toxicity due to vector dissemination to non-targeted cells that occurs even when Ad vectors are locally administered to the tissue of interest. Another hurdle is that several cell types are refractory to adenoviral infection mainly due to the lack of sufficient expression of the coxsackie-adenovirus receptor (CAR). Such cells include for example advanced cancer cells and hematopoietic or neural stem cells, where extremely high vector doses are required to achieve efficient gene transfer to these cell types. This in turn increases unwanted side effects, such as

vector-associated immunogenic toxicities. These restrictions can be circumvented through cell directed (tropism-modifying) strategies (Douglas et al. 1996; Alemany and Curiel 2001; Dorer and Nettelbeck 2009). To understand the biotechnological processes required to generate Ad vectors with selective tropism, the principle mechanism of adenovirus uptake as a two-stage process with two major subsequent interactions between capsid proteins and cell surface receptors needs to be addressed. Initially, the trimeric carboxy terminal knob domain of the viral fiber protein interacts with CAR (Bergelson et al. 1997; Miller et al. 1998; Roelvink et al. 1998; Myhre et al. 2007). After fiber-cell attachment the RGD-motif in the penton base interacts directly with cellular $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, thereby triggering internalization of the virus (Wickham et al. 1993). Native entry mechanisms and key features of unmodified viral vectors are summarized by Waehler and colleagues (2007). In contrast, the transduction efficiency of Ad is substantially diminished when CAR and αν integrin is absent on target cells (Wickham et al. 1993, 1995).

Targeting Ad vector-mediated gene transfer to a cell type or tissue of interest can be achieved by modifying the virus tropism in several ways (Kreppel et al. 2005; Kreppel and Kochanek 2008). One approach is based on coupling peptide sequences to the virus surface aiming at widening its tropism (Wickham 2000). This is extremely considerable in cancer therapy since many tumor cells do not express CAR and thus are resistant to Ad gene therapy (Miller et al. 1998). Another example are neural stem cells where analysis of Ad receptor expression revealed a complete lack of CAR and no or low expression of αν- and β_5-integrins (Schmidt et al. 2005). A second way for widening the viral tropism is the use of bispecific antibodies or bifunctional adaptor molecules composed of an anti-fiber antibody fragment and a binding component for a cell specific receptor or second antibody conjugated with specific cell surface antigens. In a first demonstration of a CAR-independent targeting, Fab-folate conjugate was attached to the fiber knob of Ad and shown to efficiently direct Ad infection of target cells via the folate receptor (Douglas et al. 1999). In a similar strategy, a conjugated fibroblast growth factor (FGF) was used to target ovarian carcinoma cells (Rancourt et al. 1998), which yielded a clinical trial where FGF2-conjugated Ad vector expressing human herpes simplex virus TK was applied in patients (Bauerschmitz et al. 2002a). Reynolds et al. (2000) succeeded in targeting pulmonary endothelial cells in vivo by intravenous injection of Ad vectors complexed with bispecific antibody against the Ad fiber knob and angiotensin-converting enzyme. Another approach was developed by Watkins and colleagues (1997) with a construct that encodes a fusion protein composed of a neutralizing anti-adenovirus fiber single-chain antibody (scFv S11) fused to a specific ligand directed against cellular receptors, termed “adenobody”. Coating virus with this adenobody ablates CAR binding and directs the particle to the desired cellular receptor. ScFvS11 can be produced in eukaryotic as well as prokaryotic cells. Due to a 6-His-tag sequence, purification and concentration of the fusion protein can be easily performed by nickel-affinity chromatography. This procedure ensures high yields of pure protein without loss of activity for in vivo and in vitro studies. Bispecific constructs directing Ad fibers to cells were developed for epidermal growth factor receptor (van Beusechem et al. 2002; Haisma et al. 2000), human endoglin (Nettelbeck et al. 2001), a specific

melanoma cell line receptor (Nettelbeck et al. 2004), endothelial receptors (Haisma et al. 2010) and the lymphocyte antigen six complex (van Zeeburg et al. 2010). The S11 approach should ultimately lead to successful systemic applications although results in animal models are still limited. A rather elegant approach uses a soluble truncated form of CAR as the virus attachment site fused to human epidermal growth factor (EGF) to direct the vector against cancer cells that express the EGF-receptor (Dmitriev et al. 2000; Hemminki et al. 2001; Kashentseva et al. 2002). Disadvantages of pure adaptor based systems in vivo are an exclusive expansion of the viral tropism leading to unspecific binding to different organs or tissues via its natural tropism. In particular, vector accumulation in the liver is unavoidable. After systemic administration normally more than 80% of circulating Ad vectors are sequestered in the liver because of a marked hepatotropism and therefore, do not reach their target (Huard et al. 1995; Reynolds et al. 2001a; Shayakhmetov et al. 2004).

An alternative strategy to develop strictly targeted adenovirus vectors is by genetic modification of the viral capsid, especially of the fiber-knob-domain by deleting the natural binding sites (Einfeld et al. 2001). In recent years, different Ad vectors were manipulated by altering the CAR attachment motif in the fiber protein as well as the RGD internalization motif in the penton base (Alemany and Curiel 2001; Leissner et al. 2001; Mizuguchi et al. 2002; Smith et al. 2002; Glasgow et al. 2006). These combined mutations almost completely eliminated vector transduction of specific neural cell types (Thomas et al. 2002). A further step of development in the application of vectors depleted for their native tropism was the identification and integration of a new tropism for instance a peptide ligand with high affinity for a specific cellular receptor at suitable insertion site of the Ad vector capsid, which does not impair virion assembly (Michael et al. 1995; Wickham et al. 1996, 1997; Hidaka et al. 1999). In this regard, integration of foreign short sequences like RGD or heparinsulfat-binding polylysine has shown great promise, whereas genetic modification by longer sequences led to inefficient packaging and waste of the viral trimerisation signal (Wickham et al. 1997). The HI-loop in the fiber knob domain was identified as a suitable integration site for new cell receptor ligands (Dmitriev et al. 1998; Krasnykh et al. 1998; Mizuguchi et al. 2001; Belousova et al. 2002; Bilbao et al. 2002; Nettelbeck et al. 2004; Rein et al. 2004). On that score, Nicklin and colleagues reported successful incorporation of a short peptide sequence in the HI-loop, which binds selectively to endothelial cells and led to an increase in transduction of endothelia (Nicklin et al. 2001a). For this purpose, the importance of the insertion site of the ligand was demonstrated when introducing the model peptide CDCRGDCFC into the knob (Hesse et al. 2007). Substitution of fiber knob tropism can be driven by introducing shaft domains of other adenovirus serotypes as well as integration of an appropriate target sequence and trimerization signal (Magnusson et al. 2001; von Seggern et al. 1999; Kanerva et al. 2002; Nakamura et al. 2003; Vigne et al. 2003). Vectors entirely based on the Ad35 serotype will be useful for selective gene transfer via CD46, which is often upregulated in tumors. Vectors chimeric for fiber and/or penton proteins such as Ad5/f35, Ad5/p35/f35 efficiently

accumulate in the lung instead of the liver (Shayakhmetov and Lieber 2000; Mercier et al. 2004; Hedley et al. 2006; Greig et al. 2009).

Myhre and colleagues used a fiber-deleted adenovirus carrying a so called "affibody" molecule, based on the B domain of Staphylococcal protein A, to selectively transduce the desired cells. This vector is constructed by replacing the knob by an extrinsic trimerization signal and the affibody (Myhre et al. 2007, 2009). In many anti-cancer approaches affibodies were specifically designed against the epidermal growth factor receptor type 2, a major molecular marker of human tumors (Belousova et al. 2008; Magnusson et al. 2012).

19.2.2 Selective Expression Using Adenoviral Vectors

Besides the above described vector shell manipulations, other possibilities exist for genetic cell targeting. One of the most versatile approaches is transcriptional targeting, which refers to the usage of tissue-specific promoters that restrict therapeutic gene expression to specific cell types. Transcriptional targeting, for example endothelial cells in neoplasm, involves the application of tumor-specific promoters for selective expression of therapeutic genes (Nicklin et al. 2001b; Reynolds et al. 2001b; Bauerschmitz et al. 2002b; Savontaus et al. 2002; Glasgow et al. 2006). The promoters, vectors, and therapeutic genes that have been utilized for transcriptional targeting of tumor endothelial cells are summarized by Dong and Nor (2009). As a further development, conditionally replicating adenoviruses (CRAds) are designed to selectively replicate in cancer cells and to subsequently lyse them. This approach is also known as virotherapy. A combination therapy with CRAds and cell specific promoters can enhance a specific transgene expression in target cell, showing a synergistic antitumor effect (Alemany et al. 2000; Haviv and Curiel 2003; Senac et al. 2010; Jiang et al. 2011; Kim et al. 2012). Viral oncolysis represents a treatment modality that offers together with tumor specific promoter driven transgene expression a unique tumor targeting opportunity (Dorer and Nettelbeck 2009). The highest level of specificity, however, to concentrate transgene expression in target cells may certainly be accomplished by combining physical and transcriptional targeting strategies within a single vector. This has been demonstrated by the combination of fiber modification e.g. RGD insertion, or fusion of a chimeric fiber motif and the CD4 ligand with the cell specific promoter VEGFR-1 (Izumi et al. 2005; Kaliberov et al. 2005; Dong and Nor 2009) or dual targeting of cancer with a tumor-specific promoter (Barnett et al. 2002a), where highly selective transgene expression was reached in the target cells. To target healthy cells, transcriptional targeting with different mammalian cellular promoters was used to restrict transgene expression to neurons, glia or undifferentiated cells (Boulaire et al. 2009). In addition, conditional gene expression in the adult mouse brain has been achieved by a combination of viral and Cre/loxP technologies (Sinnayah et al. 2004).

19.3 Technological and Biological Opportunities for Therapeutic Devices

19.3.1 Screening of Cell-Specific Peptides

Peptides possess appropriate properties to serve as cell targeting agents and are useful alternatives to antibody-based approaches since cell-specific receptors are often unknown. The phage display technology has been used to identify tissue or cell specific ligands in cell culture and in vivo (Fig. 19.1). As early as 1990, researchers constructed an epitope library where more than 10^9 peptides are expressed as a conglomerate of filamentous phage clones, each displaying one peptide sequence on the virion surface. The amino acid sequences of the peptides exposed on the phage were defined by sequencing the corresponding coding region in the phage DNA (Scott and Smith 1990). The display of polypeptide repertoires on the surface of

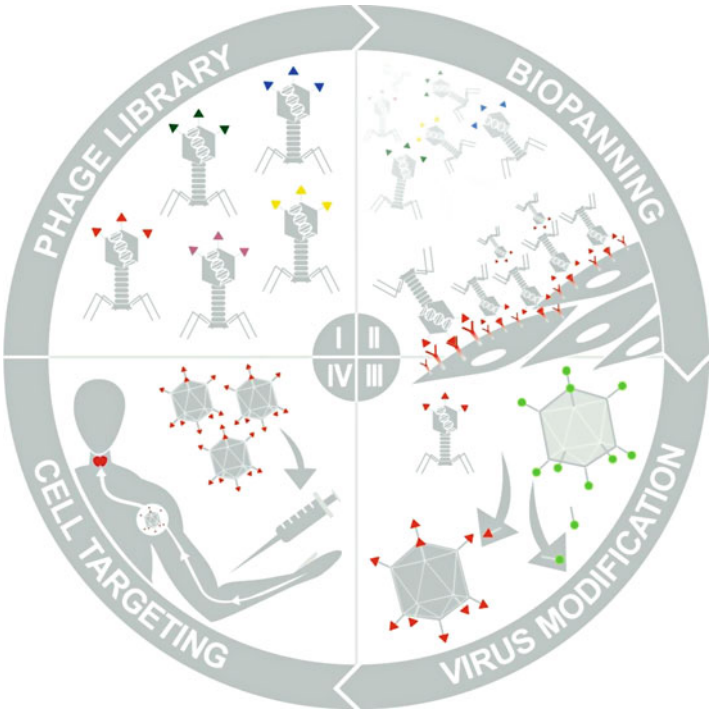


Fig. 19.1 Identification of target cell specific peptide ligands by phage display technique. A phage library of 2×10^9 peptide sequences is incubated with target cells (I). Bound phages are eluated and amplified in *E. coli*. After several rounds of biopanning (II) best binding phages are sequenced and tested for their cell binding affinity and specificity in vitro and in vivo. Target cell selective peptides are subsequently linked to modified Ad vector (III) that can be utilized for targeted gene delivery following systemic injection (IV)

phages, together with the efficient selection and amplification of the desired binding specificities was then shown to be an efficient route towards isolation of specific peptides that can act as vehicles for targeting applications (Nicklin et al. 2000; Essler and Ruoslahti 2002; Dias-Neto et al. 2009). Phage display was performed to screen for specific ligands on animal tissues (Pasqualini et al. 1997; Rajotte et al. 1998; Ruoslahti and Rajotte 2000; Spear et al. 2001; Joyce et al. 2003; Rafii et al. 2003; Lee et al. 2004; Nowakowski et al. 2004; Su et al. 2005; Di Niro et al. 2007; Giordano et al. 2009). This technique was successfully employed to obtain peptides that specifically recognize and bind normal and diseased tissue like vascular endothelium (Pasqualini and Ruoslahti 1996; Arap et al. 1998; Pasqualini et al. 2000; Arap and Pasqualini 2001; White et al. 2001; Tamm et al. 2003), lymphatic vessels (Laakkonen et al. 2002), kidney tubules (Odermatt et al. 2001), and several others (Barry et al. 1996; Ravera et al. 1998; Ivanenkov et al. 1999; Mazzucchelli et al. 1999; Nicklin et al. 2000). Many novel peptides homing to angiogenic vessels showed relatively specific affinity with several tumor types (reviewed by Liu and Wu 2008). Furthermore, the lack of gene transfer systems that are effective in selectively targeting cancer tissues prompted the search for tumor-specific peptide molecules of unknown tumor-associated receptors. In this regard, we did biopanning on human medullary thyroid carcinoma (MTC) cells in vitro (Fig. 19.1) and of transplanted tumor xenografts in vivo. The selected phages showed highly specific binding and internalization of tumor cells in culture and after systemic injection in nude mice (Böckmann et al. 2005a). In addition, a complex phage display peptide library was intravenously injected into RET-C634R transgenic mice carrying orthotopic MTCs (Böckmann et al. 2005b). Systemic administration of the identified phage resulted in a specific binding to the tumor, whereas binding to other organs such as liver was considerably reduced (up to 90%). Notably, the peptide ligand identified in the murine MTC model also selectively targeted human MTC cells in culture and under in vivo conditions (Böckmann et al. 2005b), suggesting abundant expression of its cognate receptor in murine and human medullary thyroid carcinoma. Beyond that, researchers did the first step in developing a molecular map of human vasculature by screening a peptide library in a patient (Arap et al. 2002; Chang et al. 2009; Seung-Min et al. 2009).

19.3.2 Properties of Peptide Targeted Ad Vectors

A peptide-directed Ad vector tropism to a corresponding receptor on target cells can generally be achieved by genetic integration of the peptide sequence into the viral capsid. A disadvantage of peptides that are identified by phage display libraries is that they are often functionally inactive when integrated into the fiber knob of Ad vectors. Peptides inserted into the HI-loop of the fiber knob are constrained at both, the N- and C-termini, whereas peptides inserted at the C-terminus of the fiber knob are constrained only at the N-terminus. In contrast, peptides identified by filamentous phage display libraries are constrained only at the C-terminus, when the

peptides are displayed as a fusion protein with the product of gene III of the phage. Furthermore, the lack of efficacy would be dependent on conformational changes after insertion of the peptide into the fiber knob.

To overcome these limitations, optimized transduction by a targeted Ad vector can be achieved by linking cell specific peptides to the surface of adenovirus, which is completely ablated of its native receptor interactions (Douglas et al. 1996; Peng and Russell 1999; van Beusechem et al. 2002). Such vectors are internalized into target cells independently of the native viral tropism. Since the fiber knob binds to CAR, this interaction must be abolished first. Mutation of the AB, DE, or FG loop of the fiber knob has been reported to ablate the fiber-CAR interaction (Bewley et al. 1999; Kirby et al. 1999; Roelvink et al. 1999). These mutations of the fiber knob greatly reduce the transduction efficiency of Ad vectors to CAR-positive cells in vitro. In addition, the rather minor interaction of the RGD-motif of the penton base with the αv-Intergrin receptor must be depleted as well (Mizuguchi et al. 2002). Whereas the double mutation markedly reduces retention of the vector in the liver (Einfeld et al. 2001; Koizumi et al. 2003), single mutations in the fiber knob or penton base do not change the biodistribution of Ad vectors in mice after injection (Nakamura et al. 2003; Smith et al. 2002, 2003a, b; Alemany and Curiel 2001; Leissner et al. 2001)

A common strategy to couple cell specific peptides to otherwise ablated Ad vectors is done by chemical conjugation with polyethylene glycol (PEG). Activated PEG reacts preferentially with free lysine residues on the viral surface of Ad capsid (O'Riordan et al. 1999; Romanczuk et al. 1999; Alemany et al. 2000; Croyle et al. 2002; Lanciotti et al. 2003; Eto et al. 2004; Ogawara et al. 2004; Hofherr et al. 2008; Wonganan and Croyle 2010). In general, Ad vector PEGylation was shown to significantly reduce innate immune responses (Croyle et al. 2005; Mok et al. 2005), hepatotoxic side effects (Gao et al. 2007), cytokine secretion and toxicity, and prolongs the vectors plasma half-life (Wonganan et al. 2011). On the other site, one hindrance of vector PEGylation is the decreased efficiency of infection due to steric hindrance by PEG chains (O'Riordan et al. 1999; Alemany et al. 2000; Lanciotti et al. 2003; Eto et al. 2004, 2005, 2010; Ogawara et al. 2004; Croyle et al. 2005). This can be overcome by the covalent binding of PEG to a peptide molecule. For instance, coupling of a short RGD motif on the tip of PEG has shown both high transduction efficiency in vitro (Lanciotti et al. 2003; Eto et al. 2004, 2005; Ogawara et al. 2004) as well as improvement of systemic gene delivery (Xiong et al. 2006; Gao et al. 2007). In a model of ovarian cancer, a PEGylated Ad vector was linked to fibroblast growth factor 2. This vector mediated increased transgene expression in tumor tissue and reduced localization of adenovirus to non-target cells compared to unmodified Ad (Lanciotti et al. 2003). Other studies used a multivalent hydrophilic polymer based on poly[N-(2-hydroxypropyl)methacrylamide] instead of PEG (Fisher et al. 2001; Green et al. 2004). Although bifunctional polymers like PEG are highly suitable for coating peptides to the viral surface, the success of such approaches might depend on the length of peptides. Regarding gene therapy of metastatic cancer by Ad vectors, a dual cancer-specific targeting vector system was used: PEGylation combined with the telomere reverse transcriptase promoter. The

aim was to treat experimental metastases through systemic administration of the vectors. The authors, however, summarized that the system is limited since systemic administration of Ad vectors yields low therapeutic but severe side effects (Yao et al. 2009).

19.3.3 New Prospects of "Smart" Ad Vectors for Regenerative CNS Therapies

A main focus of attention lies in the development of therapies against degenerative disorders of the central nervous system (CNS) and neoplasms of the brain with viral vectors (Arnhold et al. 2002, 2003; Deglon and Hantraye 2005; Blurton-Jones et al. 2009). The properties of neural stem/precursor cells (NSC) in the adult brain make them potentially suitable for restorative cell replacement strategies as well as delivery vehicles for gene therapy (Müller et al. 2006). NSC are found throughout the entire adult brain (Palmer et al. 1999) but they generate neurons only in two specialized niches: the subgranular zone (SGZ) in the hippocampal dentate gyrus (Palmer et al. 1997; Filippov et al. 2003) and the side wall of the lateral ventricles, the subventricular zone (SVZ) (Doetsch and Alvarez-Buylla 1996). Due to their migratory properties, the cells are attracted to brain lesions including areas of neurodegeneration and brain malignancies. For example, they have shown tropism for gliomas and degenerating spinal cord motor neurons in amyotrophic lateral sclerosis transgenic mice (Glass et al. 2005; Chi et al. 2006). Failing adult hippocampal neurogenesis has been brought into connection with the pathogenesis of disorders as diverse as dementia, major depression, and temporal lobe epilepsy (Parent et al. 2002; Kempermann et al. 2003; Steiner et al. 2006). The potential for the treatment of CNS disorders including those affecting the hippocampus has tremendously advanced with the ability to identify specific genes whose defect or absence is responsible for the particular pathological condition (Ryu et al. 2005) and genes that control precursor cell differentiation (Scheel et al. 2005). Successful delivery of these therapeutic genes to stem/precursor cells will thus provide a significant advancement in therapy for certain brain disorders. So far, experimental cell therapy for CNS disorders has been based on the transplantation of ex vivo expanded and genetically engineered NSC. The normal course of NSC development and migration in vivo is, however, controlled by the microenvironment in the neurogenic regions of the brain. Because culture conditions strongly influence the phenotype of cells, cultivation could markedly alter the cellular response to their environment once they are reintroduced into the organism. A promising option for utilizing the therapeutic potential of endogenous NSC in the brain, and most notably the hippocampus, is by application of a NSC-specific gene delivery system. Currently, the most efficient and popular way of introducing genes into NSC is by means of lentiviral vectors (Geraerts et al. 2006). It should be noted however, that NSC transduced with oncolytic Ad virus in an orthotopic xenograft model of human glioma are able to inhibit tumor growth and increase median survival by 50 % (Ahmed et al. 2011). On the basis of these

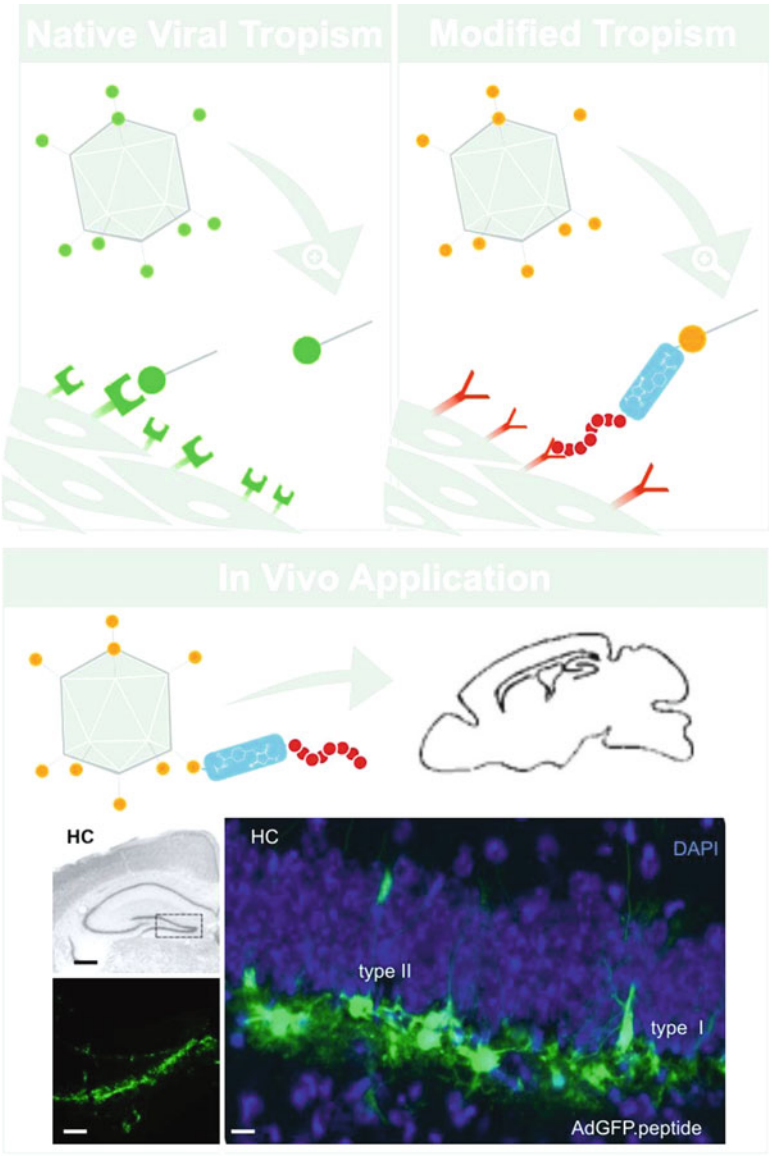


Fig. 19.2 Administration of peptide-tagged Ad vectors into the brain of adult mice. Redirection of an Ad vector ablated of its native tropism (mutated CAR and RGD motif) to adult neural stem/precursor cells was achieved by coupling of a cell specific peptide via crosslinker (or PEG) to viral surface protein. After direct injection of the virus AdGFP.peptide (expressing green fluorescent protein) into the hippocampus, specifically labeled type I and type II NSC (*green*) are visible in the dentate gyrus (*blue*: DAPI staining)

previous studies, we focused on the establishment of stem cell specific Ad vectors to genetically manipulate or reprogram neural stem cells in situ (Schmidt et al. 2007).

Early stage cultures of primary neurospheres established from the hippocampal area of adult mice were incubated with a 7mer phage library that offers the possibility to identify small peptides. Neurospheres contain only a few true stem cells and show differentiation at the core, making them a heterogeneous cluster of cells (Reynolds and Rietze 2005; Siebzehnrubl et al. 2011). To address this issue, we used neurospheres at an early stage, when a large proportion of the cells is proliferative and expresses precursor cell marker nestin. In addition, we designed a two-stage experiment: we first used cultured cells to identify peptides with presumed high specificity. We then tested these peptides in vivo to confirm their sensitivity with immunohistological tools and according to the criteria developed to identify precursor cells in the neurogenic regions of the adult hippocampus (Kempermann et al. 2004a, b; Steiner et al. 2006). In vitro selected peptides showed efficient and specific binding and uptake to cultured hippocampal neurospheres. Analysis of adenoviral distribution after vector injection into the adult mouse brain revealed a highly specific infection of type-1 and type-2 precursors (Hildebrandt et al. 2010) in the granule cell layer of the hippocampal dentate gyrus when capsid-mutated Ad vectors displaying the specific peptides were used (Fig. 19.2), whereas Ad with the wild-type capsid also transduced other differentiated brain cells. Apart from the notable target cell specificity of NSC-peptide tagged Ad vectors, infection of stem/precursor cells was confined to the brain region where NSC (used for phage display) were originally isolated from, suggesting that intrinsic differences in neural stem cells between different brain regions can translate into a diverse spectrum of cell surface receptors on these cells. Consequently, our findings imply that NSC-specific Ad vectors are needed for individual neurogenic regions. Overall, our approach represents the first opportunity for specific labeling and manipulation of NSC in the adult brain that may have major implications for monitoring stem cell development in the central nervous system and their use for cell and/or gene therapy of neurodegenerative diseases.

19.4 Applications for Therapeutic Devices

19.4.1 Adenoviral Interventions in Clinical Trials

Ad systems have remarkable advantages regarding safety issues for clinical application. In contrast to retroviral vectors, adenoviruses do not integrate into the host genome, and thereby avoid activation of oncogenes or potential elimination of tumor suppressor gene function. For many applications such as cancer gene therapy or regenerative medicine using strategies to promote stem cell differentiation or cellular proliferation, transient expression is much more desirable. Another reason for Ads being currently the most common and promising system for clinical gene therapy against cancer is due to the efficiency of gene transfer in vivo (Thomas et al. 2003; Kuhlmann et al. 2008). So far, over 400 clinical trials using Ad vectors have

been done worldwide. According to the Journal of Gene Medicine, in 2006 there were 27 studies applying Ad vectors against B-cell lymphomas, urinary bladder carcinoma, metastatic tumors, pancreatic adenocarcinoma, prostate and lung cancer. In 2011, 11 ongoing clinical phase I/II trials mainly focused on for malignant melanoma, adenocarcinoma of the lung, breast and brain cancer.

A recent phase I trial uses a tumor suppressor gene usually upregulated in prostate cancer cells which shows in vitro antitumor activity. An adenovirus expressing this gene was injected into the prostate and preliminary evidence of antitumor activity was documented (Sonpavde et al. 2011). Another trial targeting advanced digestive malignancies consists of intratumoral injection of an adenovirus encoding interleukin-12 (Mazzolini et al. 2005). With this well-tolerated approach, mild anti-tumor effects were obtained (Sangro et al. 2004). A phase I trial using an adenoviral vector containing the herpes simplex virus TK gene was applied to kill tumor cells in combination with the prodrug valacyclovir and radiation and a phase II trial of this approach is ongoing (Chiocca et al. 2011). The last described trial, a so called suicide-gene therapy (Parker et al. 2009; McBride 2012), is as well used to treat brain tumors. Furthermore, intervention of Ad vectors combined with cytostatic drugs was shown in a phase I/II trial by endoscopic intratumoral injection (Hecht et al. 2003). Moreover, intratumoral injection of an adenovirus selectively replicating in primary pancreatic tumors in a phase I trial was shown feasible and well-tolerated at doses up to 2×10^{12} particles. Intratumoral replication of the virus, however, was not detectable (Mulvihill et al. 2001). Another therapy approach used a replication-deficient adenoviral vector that expresses tumor-necrosis-factor-α under the chemotherapy- and radiation-inducible Egr-1 promoter. This study was conducted to determine the maximum tolerated dose of Ad in combination with chemoradiotherapy to treat locally advanced pancreatic cancer. Patients treated with this approach, had a better clinical outcome (Senzer et al. 2004; Hecht et al. 2012). A phase II clinical trial is focused on systemic administration of a non-replicating adenovirus vector with a pre-proendothelin-1 promoter. This promoter encodes an apoptotic receptor, which is limited to endothelial cells undergoing angiogenesis. This treatment selectively destroys tumor vascularization. Synergistic antitumor activity can be observed when combined with chemotherapy in patients with advanced solid tumors (Triozzi and Borden 2011). Four open phase III trials against refractory squamous cell carcinoma and prostate cancer have been initiated in the U.S. between 1999 till 2007 (Shirakawa 2009; Yoo et al. 2009; Nemunaitis 2011). Unresectable hepatocellular carcinoma (HCC) has unsatisfactory clinical outcomes of the patients, especially in those with recurrent HCC. H101, an E1B gene deleted adenovirus, is known to have a significant antitumor activity. H101 gene injection in combination with transarterial chemoembolization (TACE) led to a good clinical prognosis of the patient (He et al. 2011). Subsequently, 68 patients with HCC treated with TACE in combination with adenovirus-expressing p53 showed a significantly higher survival. The rAd-p53 gene therapy in combination with TACE is a safe and effective treatment modality for advanced HCC (Guan et al. 2011). Preclinical data of phase I and II trials indicate that combinations of oncolytic viruses and radiation therapy are promising tools against cancer, paving the way for several meanwhile

ongoing phase III studies (Parato et al. 2005; Wennier et al. 2011; Touchefeu et al. 2011; Hunter 2011; Hartkopf et al. 2011; Auer and Bell 2012). An example is the oncolytic virus created by genetically engineering of adenovirus, so called "Onyx-015", that allows the virus to selectively replicate in and lyse p53-deficient cancer cells.

In summary, the three main gene therapy strategies for cancer treatment are oncolytic viruses, suicide-gene therapy and gene-based immunotherapy. The first approved anticancer drug using gene therapeutic principle is the so called "Gendicine", an Ad carrying p53 tumor suppressor genes into tumor cells (Pearson et al. 2004; Peng 2005; Tani et al. 2011). Trials to correct inherited diseases such as SCID (Huston et al. 2011; Baum 2011) have achieved considerable success.

19.4.2 Barriers to Practice and Prospects

Despite the incredible progress in many aspects of Ad vector technology and the large spectrum of potential therapeutic approaches which are promising pre-clinical models, a breakthrough of Ad vector based gene therapy has not been achieved in clinical trials mainly because of several drawbacks. First generation Ad vectors activate the immune system and may evoke a strong immunological response that can potentially cause negative effects in patients (Hemminki and Alvarez 2002; Gregory et al. 2011). After the first administration of adenovirus, antibodies are developed making further administrations impossible (Arnberg 2009). Earlier generation vectors only exhibit a transient transgene expression and insufficient therapeutic effects (Dong and Nor 2009). Moreover, circulating virus primarily enters the liver, which may lead to toxic or lethal doses (Bruder and Kovesdi 1997; Lieber et al. 1997; Muruve et al. 1999; Shifrin et al. 2005). Another problem for clinical application of un-modified gene transfer systems is the lack of selectivity. This results in gene delivery not only to target cells but also to healthy environment. By application of such vectors extremely large and potentially toxic amounts of the therapeutic particles need to be administered in vivo to achieve levels good enough for a curing effect in the target tissue. Combination of several targeting strategies to circumvent such limitations improved the safety and efficacy of Ad based systems and has the potential to yield an increased therapeutic benefit in the human clinical context. Problems of inflammatory responses after Ad delivery in humans can be circumvented by immunosuppression and immunomodulation, serotype switching and microencapsulation (reviewed in detail by Bangari and Mittal 2006). Furthermore, redirecting vectors from liver towards the tissues of interest with selective cell surface receptors, a new generation of Ad chimeras, and the use of non-human serotypes might be powerful alternatives in human therapy (Barnett et al. 2002b; Di Paolo and Shayakhmetov 2009; Yao et al. 2011; Hogg et al. 2011; Schmidt et al. 2011). Further development of adenoviral vehicles like use of helper-dependent Ad vectors could bring considerable progress in clinical approaches. In comparison to earlier generations, they have a long-term transgene expression although they do not integrate in the host chromosome. In general, Ads are versatile and flexible for in vivo application,

but moderate progress in ongoing work makes further intensive investigations on vector modification essential for human regenerative medicine.

19.5 Conclusions and Future Challenges

In the last three decades, gene therapy has permanently progressed in many aspects from bench to bedside and led to enormous enhancement of potential therapeutic approaches for humans. Preclinical investigations often highlight successful treatment in animal models but so far there is a missing link to the treatment of humans in many fields of practical medicine. In clinical trials, the future of gene therapeutic interventions critically depends on the selectivity and efficiency of gene transfer to target tissues. Particularly, in vivo gene therapy of complex diseases like cancer is still problematic because of the lack of appropriate gene delivery systems (Jang et al. 2011). Impressive work on this issue shows clearly that only combinations of different cell targeting strategies lead to a selective accumulation and activity of the therapeutic gene in the tissue of interest and will allow success in treatment of patients. The development of targeted delivery systems to reach higher levels of specificity can certainly be considered as an important step towards increased therapeutic benefits for patients.

Acknowledgements The authors thank Marc Steder for preparing illustrations. Work was supported by grants from Bundesministerium für Bildung und Forschung (BMBF), Deutsche Forschungsgemeinschaft (DFG), Exzellenzförderprogramm (EFP) Mecklenburg-Vorpommern, and Medical Faculty of Rostock University (FORUN grant program).

References

Ahmed AU, Thaci B, Alexiades NG, Han Y, Qian S, Liu F, Balyasnikova IV, Ulasov IY, Aboody KS, Lesniak MS (2011) Neural stem cell-based cell carriers enhance therapeutic efficacy of an oncolytic adenovirus in an orthotopic mouse model of human glioblastoma. Mol Ther 19:1714–1726

Alemany R, Curiel DT (2001) CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. Gene Ther 8:1347–1353

Alemany R, Balague C, Curiel DT (2000) Replicative adenoviruses for cancer therapy. Nat Biotechnol 18:723–727

Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ (2011) Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol 29:341–345

Arap W, Pasqualini R (2001) The human vascular mapping project. Selection and utilization of molecules for tumor endothelial targeting. Haemostasis 31(Suppl 1):30–31

Arap W, Pasqualini R, Ruoslahti E (1998) Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279:377–380

Arap W, Kolonin MG, Trepel M, Lahdenranta J, Cardo-Vila M, Giordano RJ, Mintz PJ, Ardelt PU, Yao VJ, Vidal CI, Chen L, Flamm A, Valtanen H, Weavind LM, Hicks ME, Pollock RE, Botz GH, Bucana CD, Koivunen E, Cahill D, Troncoso P, Baggerly KA, Pentz RD, Do KA, Logothetis CJ, Pasqualini R (2002) Steps toward mapping the human vasculature by phage display. Nat Med 8:121–127

Armendariz-Borunda J, Bastidas-Ramirez BE, Sandoval-Rodriguez A, Gonzalez-Cuevas J, Gomez-Meda B, Garcia-Banuelos J (2011) Production of first generation adenoviral vectors for preclinical protocols: amplification, purification and functional titration. J Biosci Bioeng 112:415–421

Arnberg N (2009) Adenovirus receptors: implications for tropism, treatment and targeting. Rev Med Virol 19:165–178

Arnhold S, Kreppel F, Kandirali S, Lenartz D, Klinz FJ, Sturm V, Kochanek S, Andressen C, Addicks K (2002) Intracerebral transplantation and successful integration of astrocytes following genetic modification with a high-capacity adenoviral vector. Cell Transpl 11:663–670

Arnhold S, Hilgers M, Lenartz D, Semkova I, Kochanek S, Voges J, Andressen C, Addicks K (2003) Neural precursor cells as carriers for a gene therapeutical approach in tumor therapy. Cell Transpl 12:827–837

Auer R, Bell JC (2012) Oncolytic viruses: smart therapeutics for smart cancers. Future Oncol (London, England) 8:1–4

Bangari DS, Mittal SK (2006) Current strategies and future directions for eluding adenoviral vector immunity. Curr Gene Ther 6:215–226

Barnett BG, Crews CJ, Douglas JT (2002a) Targeted adenoviral vectors. Biochim Biophys Acta 1575:1–14

Barnett BG, Tillman BW, Curiel DT, Douglas JT (2002b) Dual targeting of adenoviral vectors at the levels of transduction and transcription enhances the specificity of gene expression in cancer cells. Mol Ther 6:377–385

Barry MA, Dower WJ, Johnston SA (1996) Toward cell-targeting gene therapy vectors: selection of cell-binding peptides from random peptide-presenting phage libraries. Nat Med 2:299–305

Bartel M, Schaffer D, Buning H (2011) Enhancing the clinical potential of AAV vectors by capsid engineering to evade pre-existing immunity. Front Microbiol 2:204

Bauerschmitz GJ, Lam JT, Kanerva A, Suzuki K, Nettelbeck DM, Dmitriev I, Krasnykh V, Mikheeva GV, Barnes MN, Alvarez RD, Dall P, Alemany R, Curiel DT, Hemminki A (2002a) Treatment of ovarian cancer with a tropism modified oncolytic adenovirus. Cancer Res 62:1266–1270

Bauerschmitz GJ, Nettelbeck DM, Kanerva A, Baker AH, Hemminki A, Reynolds PN, Curiel DT (2002b) The flt-1 promoter for transcriptional targeting of teratocarcinoma. Cancer Res 62:1271–1274

Baum C (2011) Gene therapy for SCID-X1: focus on clinical data. Mol Ther 19:2103–2104

Belousova N, Krendelchtchikova V, Curiel DT, Krasnykh V (2002) Modulation of adenovirus vector tropism via incorporation of polypeptide ligands into the fiber protein. J Virol 76:8621–8631

Belousova N, Mikheeva G, Gelovani J, Krasnykh V (2008) Modification of adenovirus capsid with a designed protein ligand yields a gene vector targeted to a major molecular marker of cancer. J Virol 82:630–637

Bennett J, Ashtari M, Wellman J, Marshall KA, Cyckowski LL, Chung DC, McCague S, Pierce EA, Chen Y, Bennicelli JL, Zhu X, Ying GS, Sun J, Wright JF, Auricchio A, Simonelli F, Shindler KS, Mingozzi F, High KA, Maguire AM (2012) AAV2 gene therapy readministration in three adults with congenital blindness. Sci Trans Med 4:120ra115

Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW (1997) Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275:1320–1323

Bewley MC, Springer K, Zhang YB, Freimuth P, Flanagan JM (1999) Structural analysis of the mechanism of adenovirus binding to its human cellular receptor, CAR. Science 286:1579–1583

Bilbao G, Contreras JL, Dmitriev I, Smyth CA, Jenkins S, Eckhoff D, Thomas F, Thomas J, Curiel DT (2002) Genetically modified adenovirus vector containing an RGD peptide in the HI loop of the fiber knob improves gene transfer to nonhuman primate isolated pancreatic islets. Am J Transplant 2:237–243

Bjorklund A, Kirik D, Rosenblad C, Georgievska B, Lundberg C, Mandel RJ (2000) Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. Brain Res 886:82–98

Blurton-Jones M, Kitazawa M, Martinez-Coria H, Castello NA, Müller FJ, Loring JF, Yamasaki TR, Poon WW, Green KN, LaFerla FM (2009) Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. Proc Natl Acad Sci USA 106:13594–13599

Böckmann M, Drosten M, Pützer BM (2005a) Discovery of targeting peptides for selective therapy of medullary thyroid carcinoma. J Gene Med 7:179–188

Böckmann M, Hilken G, Schmidt A, Cranston AN, Tannapfel A, Drosten M, Frilling A, Ponder BA, Pützer BM (2005b) Novel SRESPHP peptide mediates specific binding to primary medullary thyroid carcinoma after systemic injection. Hum Gene Ther 16:1267–1275

Boulaire J, Balani P, Wang S (2009) Transcriptional targeting to brain cells: engineering cell type-specific promoter containing cassettes for enhanced transgene expression. Adv Drug Deliv Rev 61:589–602

Bruder JT, Kovesdi I (1997) Adenovirus infection stimulates the Raf/MAPK signaling pathway and induces interleukin-8 expression. J Virol 71:398–404

Brunetti-Pierri N, Ng P (2008) Progress and prospects: gene therapy for genetic diseases with helper-dependent adenoviral vectors. Gene Ther 15:553–560

Brunetti-Pierri N, Ng P (2011) Helper-dependent adenoviral vectors for liver-directed gene therapy. Hum Mol Genet 20:R7–R13

Carpentier DC, Vevis K, Trabalza A, Georgiadis C, Ellison SM, Asfahani RI, Mazarakis ND (2012) Enhanced pseudotyping efficiency of HIV-1 lentiviral vectors by a rabies/vesicular stomatitis virus chimeric envelope glycoprotein. Gene Ther 19:761–764

Casper D, Engstrom SJ, Mirchandani GR, Pidel A, Palencia D, Cho PH, Brownlee M, Edelstein D, Federoff HJ, Sonstein WJ (2002) Enhanced vascularization and survival of neural transplants with ex vivo angiogenic gene transfer. Cell Transpl 11:331–349

Chailertvanitkul VA, Pouton CW (2010) Adenovirus: a blueprint for non-viral gene delivery. Curr Opin Biotechnol 21:627–632

Chang DK, Chiu CY, Kuo SY, Lin WC, Lo A, Wang YP, Li PC, Wu HC (2009) Antiangiogenic targeting liposomes increase therapeutic efficacy for solid tumors. J Biol Chem 284:12905–12916

Check E (2002) Gene therapy: shining hopes dented – but not dashed. Nature 420:735

Chi L, Ke Y, Luo C, Li B, Gozal D, Kalyanaraman B, Liu R (2006) Motor neuron degeneration promotes neural progenitor cell proliferation, migration, and neurogenesis in the spinal cords of amyotrophic lateral sclerosis mice. Stem Cells 24:34–43

Chiocca EA, Aguilar LK, Bell SD, Kaur B, Hardcastle J, Cavaliere R, McGregor J, Lo S, Ray-Chaudhuri A, Chakravarti A, Grecula J, Newton H, Harris KS, Grossman RG, Trask TW, Baskin DS, Monterroso C, Manzanera AG, Aguilar-Cordova E, New PZ (2011) Phase IB study of gene-mediated cytotoxic immunotherapy adjuvant to up-front surgery and intensive timing radiation for malignant glioma. J Clin Oncol 29:3611–3619

Clement N, Knop DR, Byrne BJ (2009) Large-scale adeno-associated viral vector production using a herpesvirus-based system enables manufacturing for clinical studies. Hum Gene Ther 20:796–806

Coffin SE (2000) Rotavirus vaccines: current controversies and future directions. Curr Infect Dis Rep 2:68–72

Coughlan L, Alba R, Parker AL, Bradshaw AC, McNeish IA, Nicklin SA, Baker AH (2010) Tropism-modification strategies for targeted gene delivery using adenoviral vectors. Viruses 2:2290–2355

Cronin J, Zhang XY, Reiser J (2005) Altering the tropism of lentiviral vectors through pseudotyping. Curr Gene Ther 5:387–398

Croyle MA, Chirmule N, Zhang Y, Wilson JM (2002) PEGylation of E1-deleted adenovirus vectors allows significant gene expression on readministration to liver. Hum Gene Ther 13:1887–1900

Croyle MA, Le HT, Linse KD, Cerullo V, Toietta G, Beaudet A, Pastore L (2005) PEGylated helper-dependent adenoviral vectors: highly efficient vectors with an enhanced safety profile. Gene Ther 12:579–587

Deglon N, Hantraye P (2005) Viral vectors as tools to model and treat neurodegenerative disorders. J Gene Med 7:530–539

Di Niro R, Ziller F, Florian F, Crovella S, Stebel M, Bestagno M, Burrone O, Bradbury AR, Secco P, Marzari R, Sblattero D (2007) Construction of miniantibodies for the in vivo study of human autoimmune diseases in animal models. BMC Biotechnol 7:46

Di Paolo NC, Shayakhmetov DM (2009) Adenovirus de-targeting from the liver. Curr Opin Mol Ther 11:523–531

Dias-Neto E, Nunes DN, Giordano RJ, Sun J, Botz GH, Yang K, Setubal JC, Pasqualini R, Arap W (2009) Next-generation phage display: integrating and comparing available molecular tools to enable cost-effective high-throughput analysis. PLoS One 4:e8338

Dmitriev I, Krasnykh V, Miller CR, Wang M, Kashentseva E, Mikheeva G, Belousova N, Curiel DT (1998) An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. J Virol 72:9706–9713

Dmitriev I, Kashentseva E, Rogers BE, Krasnykh V, Curiel DT (2000) Ectodomain of coxsackievirus and adenovirus receptor genetically fused to epidermal growth factor mediates adenovirus targeting to epidermal growth factor receptor-positive cells. J Virol 74:6875–6884

Doetsch F, Alvarez-Buylla A (1996) Network of tangential pathways for neuronal migration in adult mammalian brain. Proc Natl Acad Sci USA 93:14895–14900

Dong Z, Nor JE (2009) Transcriptional targeting of tumor endothelial cells for gene therapy. Adv Drug Deliv Rev 61:542–553

Dorer DE, Nettelbeck DM (2009) Targeting cancer by transcriptional control in cancer gene therapy and viral oncolysis. Adv Drug Deliv Rev 61:554–571

Dormond E, Kamen AA (2011) Manufacturing of adenovirus vectors: production and purification of helper dependent adenovirus. Method Mol Biol 737:139–156

Dormond E, Meneses-Acosta A, Jacob D, Durocher Y, Gilbert R, Perrier M, Kamen A (2009) An efficient and scalable process for helper-dependent adenoviral vector production using polyethylenimine-adenofection. Biotechnol Bioeng 102:800–810

Douglas JT, Rogers BE, Rosenfeld ME, Michael SI, Feng M, Curiel DT (1996) Targeted gene delivery by tropism-modified adenoviral vectors. Nat Biotechnol 14:1574–1578

Douglas JT, Miller CR, Kim M, Dmitriev I, Mikheeva G, Krasnykh V, Curiel DT (1999) A system for the propagation of adenoviral vectors with genetically modified receptor specificities. Nat Biotechnol 17:470–475

Duan D (2011) Gene delivery to the heart: an updated review on vectors and methods. J Gene Med 13:556

Einfeld DA, Schroeder R, Roelvink PW, Lizonova A, King CR, Kovesdi I, Wickham TJ (2001) Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. J Virol 75:11284–11291

Essler M, Ruoslahti E (2002) Molecular specialization of breast vasculature: a breast-homing phage-displayed peptide binds to aminopeptidase P in breast vasculature. Proc Natl Acad Sci USA 99:2252–2257

Eto Y, Gao JQ, Sekiguchi F, Kurachi S, Katayama K, Mizuguchi H, Hayakawa T, Tsutsumi Y, Mayumi T, Nakagawa S (2004) Neutralizing antibody evasion ability of adenovirus vector induced by the bioconjugation of methoxypolyethylene glycol succinimidyl propionate (MPEG-SPA). Biol Pharm Bull 27:936–938

Eto Y, Gao JQ, Sekiguchi F, Kurachi S, Katayama K, Maeda M, Kawasaki K, Mizuguchi H, Hayakawa T, Tsutsumi Y, Mayumi T, Nakagawa S (2005) PEGylated adenovirus vectors containing RGD peptides on the tip of PEG show high transduction efficiency and antibody evasion ability. J Gene Med 7:604–612

Eto Y, Yoshioka Y, Ishida T, Yao X, Morishige T, Narimatsu S, Mizuguchi H, Mukai Y, Okada N, Kiwada H, Nakagawa S (2010) Optimized PEGylated adenovirus vector reduces the anti-vector humoral immune response against adenovirus and induces a therapeutic effect against metastatic lung cancer. Biol Pharm Bull 33:1540–1544

Filippov V, Kronenberg G, Pivneva T, Reuter K, Steiner B, Wang LP, Yamaguchi M, Kettenmann H, Kempermann G (2003) Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. Mol Cell Neurosci 23:373–382

Fisher KD, Stallwood Y, Green NK, Ulbrich K, Mautner V, Seymour LW (2001) Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. Gene Ther 8:341–348

Galvez J, Lecina M, Sola C, Cairo JJ, Godia F (2012) Optimization of HEK-293S cell cultures for the production of adenoviral vectors in bioreactors using on-line OUR measurements. J Biotechnol 157:214–222

Gao JQ, Eto Y, Yoshioka Y, Sekiguchi F, Kurachi S, Morishige T, Yao X, Watanabe H, Asavatanabodee R, Sakurai F, Mizuguchi H, Okada Y, Mukai Y, Tsutsumi Y, Mayumi T, Okada N, Nakagawa S (2007) Effective tumor targeted gene transfer using PEGylated adenovirus vector via systemic administration. J Control Release 122:102–110

Gaspar HB, Cooray S, Gilmour KC, Parsley KL, Zhang F, Adams S, Bjorkegren E, Bayford J, Brown L, Davies EG, Veys P, Fairbanks L, Bordon V, Petropolou T, Kinnon C, Thrasher AJ (2011) Hematopoietic stem cell gene therapy for adenosine deaminase-deficient severe combined immunodeficiency leads to long-term immunological recovery and metabolic correction. Sci Trans Med 3:97ra80

Geraerts M, Eggermont K, Hernandez-Acosta P, Garcia-Verdugo JM, Baekelandt V, Debyser Z (2006) Lentiviral vectors mediate efficient and stable gene transfer in adult neural stem cells in vivo. Hum Gene Ther 17:635–650

Giordano RJ, Edwards JK, Tuder RM, Arap W, Pasqualini R (2009) Combinatorial ligand-directed lung targeting. Proc Am Thorac Soc 6:411–415

Glasgow JN, Everts M, Curiel DT (2006) Transductional targeting of adenovirus vectors for gene therapy. Cancer Gene Ther 13:830–844

Glass R, Synowitz M, Kronenberg G, Walzlein JH, Markovic DS, Wang LP, Gast D, Kiwit J, Kempermann G, Kettenmann H (2005) Glioblastoma-induced attraction of endogenous neural precursor cells is associated with improved survival. J Neurosci 25:2637–2646

Glorioso JC, Fink DJ (2009) Herpes vector-mediated gene transfer in the treatment of chronic pain. Mol Ther 17:13–18

Green NK, Herbert CW, Hale SJ, Hale AB, Mautner V, Harkins R, Hermiston T, Ulbrich K, Fisher KD, Seymour LW (2004) Extended plasma circulation time and decreased toxicity of polymer-coated adenovirus. Gene Ther 11:1256–1263

Gregory SM, Nazir SA, Metcalf JP (2011) Implications of the innate immune response to adenovirus and adenoviral vectors. Future Virol 6:357–374

Greig JA, Buckley SM, Waddington SN, Parker AL, Bhella D, Pink R, Rahim AA, Morita T, Nicklin SA, McVey JH, Baker AH (2009) Influence of coagulation factor x on in vitro and in vivo gene delivery by adenovirus (Ad) 5, Ad35, and chimeric Ad5/Ad35 vectors. Mol Ther 17:1683–1691

Guan YS, Liu Y, He Q, Li X, Yang L, Hu Y, La Z (2011) p53 gene therapy in combination with transcatheter arterial chemoembolization for HCC: one-year follow-up. World J Gastroenterol 17:2143–2149

Guo X, Huang L (2012) Recent advances in nonviral vectors for gene delivery. Acc Chem Res 45:971–979

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302:415–419

Haisma HJ, Grill J, Curiel DT, Hoogeland S, van Beusechem VW, Pinedo HM, Gerritsen WR (2000) Targeting of adenoviral vectors through a bispecific single-chain antibody. Cancer Gene Ther 7:901–904

Haisma HJ, Kamps GK, Bouma A, Geel TM, Rots MG, Kariath A, Bellu AR (2010) Selective targeting of adenovirus to alphavbeta3 integrins, VEGFR2 and Tie2 endothelial receptors by angio-adenobodies. Int J Pharm 391:155–161

Hartkopf AD, Fehm T, Wallwiener D, Lauer UM (2011) Oncolytic virotherapy of breast cancer. Gyn Oncol 123:164–171

Haviv YS, Curiel DT (2003) Engineering regulatory elements for conditionally-replicative adeno-viruses. Curr Gene Ther 3:357–385

He Q, Liu Y, Zou Q, Guan YS (2011) Transarterial injection of H101 in combination with chemoembolization overcomes recurrent hepatocellular carcinoma. World J Gastroenterol 17:2353–2355

Hecht JR, Bedford R, Abbruzzese JL, Lahoti S, Reid TR, Soetikno RM, Kirn DH, Freeman SM (2003) A phase I/II trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. Clin Cancer Res 9:555–561

Hecht JR, Farrell JJ, Senzer N, Nemunaitis J, Rosemurgy A, Chung T, Hanna N, Chang KJ, Javle M, Posner M, Waxman I, Reid A, Erickson R, Canto M, Chak A, Blatner G, Kovacevic M, Thornton M (2012) EUS or percutaneously guided intratumoral TNFerade biologic with 5-fluorouracil and radiotherapy for first-line treatment of locally advanced pancreatic cancer: a phase I/II study. Gastrointest Endosc 75:332–338

Hedley SJ, Chen J, Mountz JD, Li J, Curiel DT, Korokhov N, Kovesdi I (2006) Targeted and shielded adenovectors for cancer therapy. Cancer Immunol Immunother 55:1412–1419

Hemminki A, Alvarez RD (2002) Adenoviruses in oncology: a viable option? Bio Drugs 16:77–87

Hemminki A, Belousova N, Zinn KR, Liu B, Wang M, Chaudhuri TR, Rogers BE, Buchsbaum DJ, Siegal GP, Barnes MN, Gomez-Navarro J, Curiel DT, Alvarez RD (2001) An adenovirus with enhanced infectivity mediates molecular chemotherapy of ovarian cancer cells and allows imaging of gene expression. Mol Ther 4:223–231

Hesse A, Kosmides D, Kontermann RE, Nettelbeck DM (2007) Tropism modification of adenovirus vectors by peptide ligand insertion into various positions of the adenovirus serotype 41 short-fiber knob domain. J Virol 81:2688–2699

Hidaka C, Milano E, Leopold PL, Bergelson JM, Hackett NR, Finberg RW, Wickham TJ, Kovesdi I, Roelvink P, Crystal RG (1999) CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. J Clin Invest 103:579–587

Hildebrandt S, Schmidt A, Stoll A, Schmitt O, Köhling R, Wree A, Haas SJ, Pützer BM (2010) Targeting of neural stem cells in the hippocampus of adult rats by custom-made Ad vectors. Brain Struct Funct 215:105–113

Hofherr SE, Shashkova EV, Weaver EA, Khare R, Barry MA (2008) Modification of adenoviral vectors with polyethylene glycol modulates in vivo tissue tropism and gene expression. Mol Ther 16:1276–1282

Hogg RT, Thorpe P, Gerard RD (2011) Retargeting adenoviral vectors to improve gene transfer into tumors. Cancer Gene Ther 18:275–287

Hu C, Cela RG, Suzuki M, Lee B, Lipshutz GS (2011) Neonatal helper-dependent adenoviral vector gene therapy mediates correction of hemophilia A and tolerance to human factor VIII. Proc Natl Acad Sci USA 108:2082–2087

Huard J, Lochmüller H, Acsadi G, Jani A, Massie B, Karpati G (1995) The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants. Gene Ther 2:107–115

Hunter P (2011) The fourth front against cancer. The first clinical trials to test engineered viruses that attack tumour cells have yielded promising results for future cancer therapies. EMBO Rep 12:769–771

Huston MW, van Til NP, Visser TP, Arshad S, Brugman MH, Cattoglio C, Nowrouzi A, Li Y, Schambach A, Schmidt M, Baum C, von Kalle C, Mavilio F, Zhang F, Blundell MP, Thrasher AJ, Verstegen MM, Wagemaker G (2011) Correction of murine SCID-X1 by lentiviral gene therapy using a codon-optimized IL2RG gene and minimal pretransplant conditioning. Mol Ther 19:1867–1877

Ivanenkov VV, Felici F, Menon AG (1999) Targeted delivery of multivalent phage display vectors into mammalian cells. Biochim Biophys Acta 1448:463–472

Izumi M, Kawakami Y, Glasgow JN, Belousova N, Everts M, Kim-Park S, Yamamoto S, Wang M, Le LP, Reynolds PN, Curiel DT (2005) In vivo analysis of a genetically modified adenoviral vector targeted to human CD40 using a novel transient transgenic model. J Gene Med 7:1517–1525

Jafari M, Soltani M, Naahidi S, Karunaratne DN, Chen P (2012) Nonviral approach for targeted nucleic acid delivery. Curr Med Chem 19:197–208

Jang JH, Schaffer DV, Shea LD (2011) Engineering biomaterial systems to enhance viral vector gene delivery. Mol Ther 19:1407–1415
Jiang G, Xin Y, Zheng JN, Liu YQ (2011) Combining conditionally replicating adenovirus-mediated gene therapy with chemotherapy: a novel antitumor approach. Int J Cancer 129:263–274
Joyce JA, Laakkonen P, Bernasconi M, Bergers G, Ruoslahti E, Hanahan D (2003) Stage-specific vascular markers revealed by phage display in a mouse model of pancreatic islet tumorigenesis. Cancer Cell 4:393–403
Kajon AE, Erdman DD (2007) Assessment of genetic variability among subspecies b1 human adenoviruses for molecular epidemiology studies. Methods Mol Med 131:335–355
Kajon AE, Moseley JM, Metzgar D, Huong HS, Wadleigh A, Ryan MA, Russell KL (2007) Molecular epidemiology of adenovirus type 4 infections in US military recruits in the postvaccination era (1997–2003). J Infect Dis 196:67–75
Kaliberov SA, Kaliberova LN, Buchsbaum DJ (2005) Combined ionizing radiation and sKDR gene delivery for treatment of prostate carcinomas. Gene Ther 12:407–417
Kanerva A, Mikheeva GV, Krasnykh V, Coolidge CJ, Lam JT, Mahasreshti PJ, Barker SD, Straughn M, Barnes MN, Alvarez RD, Hemminki A, Curiel DT (2002) Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to ovarian cancer cells. Clin Cancer Res 8:275–280
Kashentseva EA, Seki T, Curiel DT, Dmitriev IP (2002) Adenovirus targeting to c-erbB-2 oncoprotein by single-chain antibody fused to trimeric form of adenovirus receptor ectodomain. Cancer Res 62:609–616
Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH (2003) Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. Development 130:391–399
Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004a) Milestones of neuronal development in the adult hippocampus. Trends Neurosci 27:447–452
Kempermann G, Wiskott L, Gage FH (2004b) Functional significance of adult neurogenesis. Curr Opin Neurobiol 14:186–191
Kim IH, Jozkowicz A, Piedra PA, Oka K, Chan L (2001) Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. Proc Natl Acad Sci USA 98:13282–13287
Kim DR, Park MY, Lim HJ, Park JS, Cho YJ, Lee SW, Yoon HI, Lee JH, Kim YS, Lee CT (2012) Combination therapy of conditionally replicating adenovirus and histone deacetylase inhibitors. Int J Mol Med 29:218–224
Kirby I, Davison E, Beavil AJ, Soh CP, Wickham TJ, Roelvink PW, Kovesdi I, Sutton BJ, Santis G (1999) Mutations in the DG loop of adenovirus type 5 fiber knob protein abolish high-affinity binding to its cellular receptor CAR. J Virol 73:9508–9514
Koizumi N, Mizuguchi H, Sakurai F, Yamaguchi T, Watanabe Y, Hayakawa T (2003) Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and alphav integrin-binding ablation. J Virol 77:13062–13072
Koynova R, Tenchov B (2011) Recent patents in cationic lipid carriers for delivery of nucleic acids. Recent Pat DNA Gene Seq 5:8–27
Krasnykh V, Dmitriev I, Mikheeva G, Miller CR, Belousova N, Curiel DT (1998) Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. J Virol 72:1844–1852
Kreppel F, Kochanek S (2008) Modification of adenovirus gene transfer vectors with synthetic polymers: a scientific review and technical guide. Mol Ther 16:16–29
Kreppel F, Gackowski J, Schmidt E, Kochanek S (2005) Combined genetic and chemical capsid modifications enable flexible and efficient de- and retargeting of adenovirus vectors. Mol Ther 12:107–117
Kuhlmann KF, Gouma DJ, Wesseling JG (2008) Adenoviral gene therapy for pancreatic cancer: where do we stand? Dig Surg 25:278–292
Laakkonen P, Porkka K, Hoffman JA, Ruoslahti E (2002) A tumor-homing peptide with a targeting specificity related to lymphatic vessels. Nat Med 8:751–755

Lanciotti J, Song A, Doukas J, Sosnowski B, Pierce G, Gregory R, Wadsworth S, O'Riordan C (2003) Targeting adenoviral vectors using heterofunctional polyethylene glycol FGF2 conjugates. Mol Ther 8:99–107

Lee TY, Wu HC, Tseng YL, Lin CT (2004) A novel peptide specifically binding to nasopharyngeal carcinoma for targeted drug delivery. Cancer Res 64:8002–8008

Leissner P, Legrand V, Schlesinger Y, Hadji DA, van Raaij M, Cusack S, Pavirani A, Mehtali M (2001) Influence of adenoviral fiber mutations on viral encapsidation, infectivity and in vivo tropism. Gene Ther 8:49–57

Lieber A, He CY, Meuse L, Schowalter D, Kirillova I, Winther B, Kay MA (1997) The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. J Virol 71:8798–8807

Liu Z, Wu K (2008) Peptides homing to tumor vasculature: imaging and therapeutics for cancer. Recent Pat Anticancer Drug Discov 3:202–208

Lotti F, Menguzzato E, Rossi C, Naldini L, Ailles L, Mavilio F, Ferrari G (2002) Transcriptional targeting of lentiviral vectors by long terminal repeat enhancer replacement. J Virol 76:3996–4007

Magnusson MK, Hong SS, Boulanger P, Lindholm L (2001) Genetic retargeting of adenovirus: novel strategy employing "deknobbing" of the fiber. J Virol 75:7280–7289

Magnusson MK, Kraaij R, Leadley RM, De Ridder CM, van Weerden WM, Van Schie KA, Van der Kroeg M, Hoeben RC, Maitland NJ, Lindholm L (2012) A transductionally retargeted adenoviral vector for virotherapy of Her2/neu-expressing prostate cancer. Hum Gene Ther 23:70–82

Mazzolini G, Alfaro C, Sangro B, Feijoo E, Ruiz J, Benito A, Tirapu I, Arina A, Sola J, Herraiz M, Lucena F, Olague C, Subtil J, Quiroga J, Herrero I, Sadaba B, Bendandi M, Qian C, Prieto J, Melero I (2005) Intratumoral injection of dendritic cells engineered to secrete interleukin-12 by recombinant adenovirus in patients with metastatic gastrointestinal carcinomas. J Clin Oncol 23:999–1010

Mazzucchelli L, Burritt JB, Jesaitis AJ, Nusrat A, Liang TW, Gewirtz AT, Schnell FJ, Parkos CA (1999) Cell-specific peptide binding by human neutrophils. Blood 93:1738–1748

McBride WH (2012) Integration of adenovirus thymidine kinase suicide-gene therapy with surgery and radiation therapy for malignant glioma. Future Oncol 8:17–20

Mercier GT, Campbell JA, Chappell JD, Stehle T, Dermody TS, Barry MA (2004) A chimeric adenovirus vector encoding reovirus attachment protein sigma1 targets cells expressing junctional adhesion molecule 1. Proc Natl Acad Sci USA 101:6188–6193

Michael SI, Hong JS, Curiel DT, Engler JA (1995) Addition of a short peptide ligand to the adenovirus fiber protein. Gene Ther 2:660–668

Miller CR, Buchsbaum DJ, Reynolds PN, Douglas JT, Gillespie GY, Mayo MS, Raben D, Curiel DT (1998) Differential susceptibility of primary and established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. Cancer Res 58:5738–5748

Mingozzi F, High KA (2011) Immune responses to AAV in clinical trials. Curr Gene Ther 11:321–330

Mizuguchi H, Koizumi N, Hosono T, Utoguchi N, Watanabe Y, Kay MA, Hayakawa T (2001) A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. Gene Ther 8:730–735

Mizuguchi H, Koizumi N, Hosono T, Ishii-Watabe A, Uchida E, Utoguchi N, Watanabe Y, Hayakawa T (2002) CAR- or alphav integrin-binding ablated adenovirus vectors, but not fiber-modified vectors containing RGD peptide, do not change the systemic gene transfer properties in mice. Gene Ther 9:769–776

Mok H, Palmer DJ, Ng P, Barry MA (2005) Evaluation of polyethylene glycol modification of first-generation and helper-dependent adenoviral vectors to reduce innate immune responses. Mol Ther 11:66–79

Morgenstern PF, Marongiu R, Musatov SA, Kaplitt MG (2011) Adeno-associated viral gene delivery in neurodegenerative disease. Method Mol Biol 793:443–455

Müller FJ, Snyder EY, Loring JF (2006) Gene therapy: can neural stem cells deliver? Nat Rev Neurosci 7:75–84

Mulvihill S, Warren R, Venook A, Adler A, Randlev B, Heise C, Kirn D (2001) Safety and feasibility of injection with an E1B-55 kDa gene-deleted, replication-selective adenovirus (ONYX-015) into primary carcinomas of the pancreas: a phase I trial. Gene Ther 8:308–315

Muruve DA, Barnes MJ, Stillman IE, Libermann TA (1999) Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury in vivo. Hum Gene Ther 10:965–976

Myhre S, Henning P, Granio O, Tylo AS, Nygren PA, Olofsson S, Boulanger P, Lindholm L, Hong SS (2007) Decreased immune reactivity towards a knobless, affibody-targeted adenovirus type 5 vector. Gene Ther 14:376–381

Myhre S, Henning P, Friedman M, Stahl S, Lindholm L, Magnusson MK (2009) Re-targeted adenovirus vectors with dual specificity; binding specificities conferred by two different Affibody molecules in the fiber. Gene Ther 16:252–261

Nakamura T, Sato K, Hamada H (2003) Reduction of natural adenovirus tropism to the liver by both ablation of fiber-coxsackievirus and adenovirus receptor interaction and use of replaceable short fiber. J Virol 77:2512–2521

Nemunaitis J (2011) Head and neck cancer: response to p53-based therapeutics. Head Neck 33:131–134

Nettelbeck DM, Miller DW, Jerome V, Zuzarte M, Watkins SJ, Hawkins RE, Müller R, Kontermann RE (2001) Targeting of adenovirus to endothelial cells by a bispecific single-chain diabody directed against the adenovirus fiber knob domain and human endoglin (CD105). Mol Ther 3:882–891

Nettelbeck DM, Rivera AA, Kupsch J, Dieckmann D, Douglas JT, Kontermann RE, Alemany R, Curiel DT (2004) Retargeting of adenoviral infection to melanoma: combining genetic ablation of native tropism with a recombinant bispecific single-chain diabody (scDb) adapter that binds to fiber knob and HMWMAA. Int J Cancer 108:136–145

Nicklin SA, White SJ, Watkins SJ, Hawkins RE, Baker AH (2000) Selective targeting of gene transfer to vascular endothelial cells by use of peptides isolated by phage display. Circulation 102:231–237

Nicklin SA, Reynolds PN, Brosnan MJ, White SJ, Curiel DT, Dominiczak AF, Baker AH (2001a) Analysis of cell-specific promoters for viral gene therapy targeted at the vascular endothelium. Hypertension 38:65–70

Nicklin SA, Von Seggern DJ, Work LM, Pek DC, Dominiczak AF, Nemerow GR, Baker AH (2001b) Ablating adenovirus type 5 fiber-CAR binding and HI loop insertion of the SIGYPLP peptide generate an endothelial cell-selective adenovirus. Mol Ther 4:534–542

Nowakowski GS, Dooner MS, Valinski HM, Mihaliak AM, Quesenberry PJ, Becker PS (2004) A specific heptapeptide from a phage display peptide library homes to bone marrow and binds to primitive hematopoietic stem cells. Stem Cells 22:1030–1038

Odermatt A, Audige A, Frick C, Vogt B, Frey BM, Frey FJ, Mazzucchelli L (2001) Identification of receptor ligands by screening phage-display peptide libraries ex vivo on microdissected kidney tubules. J Am Soc Nephrol 12:308–316

Ogawara K, Rots MG, Kok RJ, Moorlag HE, Van Loenen AM, Meijer DK, Haisma HJ, Molema G (2004) A novel strategy to modify adenovirus tropism and enhance transgene delivery to activated vascular endothelial cells in vitro and in vivo. Hum Gene Ther 15:433–443

O'Riordan CR, Lachapelle A, Delgado C, Parkes V, Wadsworth SC, Smith AE, Francis GE (1999) PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. Hum Gene Ther 10:1349–1358

Ortolano S, Spuch C, Navarro C (2012) Present and future of adeno associated virus based gene therapy approaches. Recent Pat Endocr Metab Immune Drug Discov 6:47–66

Pajusola K, Gruchala M, Joch H, Lüscher TF, Yla-Herttuala S, Büeler H (2002) Cell-type-specific characteristics modulate the transduction efficiency of adeno-associated virus type 2 and restrain infection of endothelial cells. J Virol 76:11530–11540

Palmer TD, Takahashi J, Gage FH (1997) The adult rat hippocampus contains primordial neural stem cells. Mol Cell Neurosci 8:389–404

Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH (1999) Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J Neurosci 19:8487–8497

Parato KA, Senger D, Forsyth PA, Bell JC (2005) Recent progress in the battle between oncolytic viruses and tumours. Nat Rev Cancer 5:965–976

Parent JM, Valentin VV, Lowenstein DH (2002) Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone-olfactory bulb pathway. J Neurosci 22:3174–3188

Parker JN, Bauer DF, Cody JJ, Markert JM (2009) Oncolytic viral therapy of malignant glioma. Neurotherapeutics 6:558–569

Parks RJ (2000) Improvements in adenoviral vector technology: overcoming barriers for gene therapy. Clin Genet 58:1–11

Pasqualini R, Ruoslahti E (1996) Organ targeting in vivo using phage display peptide libraries. Nature 380:364–366

Pasqualini R, Koivunen E, Ruoslahti E (1997) Alpha v integrins as receptors for tumor targeting by circulating ligands. Nat Biotechnol 15:542–546

Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A, Ashmun RA, Shapiro LH, Arap W, Ruoslahti E (2000) Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res 60:722–727

Patil SD, Rhodes DG, Burgess DJ (2005) DNA-based therapeutics and DNA delivery systems: a comprehensive review. AAPS J 7:E61–E77

Pearson S, Jia H, Kandachi K (2004) China approves first gene therapy. Nat Biotechnol 22:3–4

Peng Z (2005) Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. Hum Gene Ther 16:1016–1027

Peng KW, Russell SJ (1999) Viral vector targeting. Curr Opin Biotechnol 10:454–457

Preuss MA, Curiel DT (2007) Gene therapy: science fiction or reality? South Med J 100:101–104

Qiu J, Handa A, Kirby M, Brown KE (2000) The interaction of heparin sulfate and adeno-associated virus 2. Virology 269:137–147

Rafii S, Avecilla ST, Jin DK (2003) Tumor vasculature address book: identification of stage-specific tumor vessel zip codes by phage display. Cancer Cell 4:331–333

Rajotte D, Arap W, Hagedorn M, Koivunen E, Pasqualini R, Ruoslahti E (1998) Molecular heterogeneity of the vascular endothelium revealed by in vivo phage display. J Clin Invest 102:430–437

Rancourt C, Rogers BE, Sosnowski BA, Wang M, Piche A, Pierce GF, Alvarez RD, Siegal GP, Douglas JT, Curiel DT (1998) Basic fibroblast growth factor enhancement of adenovirus-mediated delivery of the herpes simplex virus thymidine kinase gene results in augmented therapeutic benefit in a murine model of ovarian cancer. Clin Cancer Res 4:2455–2461

Ravera MW, Carcamo J, Brissette R, Alam-Moghe A, Dedova O, Cheng W, Hsiao KC, Klebanov D, Shen H, Tang P, Blume A, Mandecki W (1998) Identification of an allosteric binding site on the transcription factor p53 using a phage-displayed peptide library. Oncogene 16:1993–1999

Rein DT, Breidenbach M, Wu H, Han T, Haviv YS, Wang M, Kirby TO, Kawakami Y, Dall P, Alvarez RD, Curiel DT (2004) Gene transfer to cervical cancer with fiber-modified adenoviruses. Int J Cancer 111:698–704

Reynolds BA, Rietze RL (2005) Neural stem cells and neurospheres–re-evaluating the relationship. Nat Methods 2:333–336

Reynolds PN, Zinn KR, Gavrilyuk VD, Balyasnikova IV, Rogers BE, Buchsbaum DJ, Wang MH, Miletich DJ, Grizzle WE, Douglas JT, Danilov SM, Curiel DT (2000) A targetable, injectable adenoviral vector for selective gene delivery to pulmonary endothelium in vivo. Mol Ther 2:562–578

Reynolds PN, Holmes MD, Adachi Y, Kaliberova L, Curiel DT (2001a) A novel system for mitigation of ectopic transgene expression induced by adenoviral vectors. Gene Ther 8:1271–1275

Reynolds PN, Nicklin SA, Kaliberova L, Boatman BG, Grizzle WE, Balyasnikova IV, Baker AH, Danilov SM, Curiel DT (2001b) Combined transductional and transcriptional targeting improves the specificity of transgene expression in vivo. Nat Biotechnol 19:838–842

Roelvink PW, Lizonova A, Lee JG, Li Y, Bergelson JM, Finberg RW, Brough DE, Kovesdi I, Wickham TJ (1998) The coxsackievirus-adenovirus receptor protein can function as a cellular

attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. J Virol 72:7909–7915
Roelvink PW, Mi Lee G, Einfeld DA, Kovesdi I, Wickham TJ (1999) Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. Science 286:1568–1571
Romanczuk H, Galer CE, Zabner J, Barsomian G, Wadsworth SC, O'Riordan CR (1999) Modification of an adenoviral vector with biologically selected peptides: a novel strategy for gene delivery to cells of choice. Hum Gene Ther 10:2615–2626
Ruoslahti E, Rajotte D (2000) An address system in the vasculature of normal tissues and tumors. Annu Rev Immunol 18:813–827
Ryu EJ, Angelastro JM, Greene LA (2005) Analysis of gene expression changes in a cellular model of Parkinson disease. Neurobiol Dis 18:54–74
Sangro B, Mazzolini G, Ruiz J, Herraiz M, Quiroga J, Herrero I, Benito A, Larrache J, Pueyo J, Subtil JC, Olague C, Sola J, Sadaba B, Lacasa C, Melero I, Qian C, Prieto J (2004) Phase I trial of intratumoral injection of an adenovirus encoding interleukin-12 for advanced digestive tumors. J Clin Oncol 22:1389–1397
Savontaus MJ, Sauter BV, Huang TG, Woo SL (2002) Transcriptional targeting of conditionally replicating adenovirus to dividing endothelial cells. Gene Ther 9:972–979
Schambach A, Galla M, Modlich U, Will E, Chandra S, Reeves L, Colbert M, Williams DA, von Kalle C, Baum C (2006) Lentiviral vectors pseudotyped with murine ecotropic envelope: increased biosafety and convenience in preclinical research. Exp Hematol 34:588–592
Scheel JR, Ray J, Gage FH, Barlow C (2005) Quantitative analysis of gene expression in living adult neural stem cells by gene trapping. Nat Methods 2:363–370
Schmidt A, Böckmann M, Stoll A, Racek T, Pützer BM (2005) Analysis of adenovirus gene transfer into adult neural stem cells. Virus Res 114:45–53
Schmidt A, Haas SJ, Hildebrandt S, Scheibe J, Eckhoff B, Racek T, Kempermann G, Wree A, Pützer BM (2007) Selective targeting of adenoviral vectors to neural precursor cells in the hippocampus of adult mice: new prospects for in situ gene therapy. Stem Cells 25:2910–2918
Schmidt A, Eipel C, Fürst K, Sommer N, Pahnke J, Pützer BM (2011) Evaluation of systemic targeting of RET oncogene-based MTC with tumor-selective peptide-tagged Ad vectors in clinical mouse models. Gene Ther 18:418–423
Scott JK, Smith GP (1990) Searching for peptide ligands with an epitope library. Science 249:386–390
Senac JS, Doronin K, Russell SJ, Jelinek DF, Greipp PR, Barry MA (2010) Infection and killing of multiple myeloma by adenoviruses. Hum Gene Ther 21:179–190
Senzer N, Mani S, Rosemurgy A, Nemunaitis J, Cunningham C, Guha C, Bayol N, Gillen M, Chu K, Rasmussen C, Rasmussen H, Kufe D, Weichselbaum R, Hanna N (2004) TNFerade biologic, an adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene: a phase I study in patients with solid tumors. J Clin Oncol 22:592–601
Seow Y, Wood MJ (2009) Biological gene delivery vehicles: beyond viral vectors. Mol Ther 17:767–777
Seung-Min L, Gil-Suk Y, Eun-Sang Y, Tae-Gyun K, In-San K, Byung-Heon L (2009) Application of phage display to discovery of tumor-specific homing peptides: developing strategies for therapy and molecular imaging of cancer. Method Mol Biol 512:355–363
Shayakhmetov DM, Lieber A (2000) Dependence of adenovirus infectivity on length of the fiber shaft domain. J Virol 74:10274–10286
Shayakhmetov DM, Li ZY, Ni S, Lieber A (2004) Analysis of adenovirus sequestration in the liver, transduction of hepatic cells, and innate toxicity after injection of fiber-modified vectors. J Virol 78:5368–5381
Shichinohe T, Bochner BH, Mizutani K, Nishida M, Hegerich-Gilliam S, Naldini L, Kasahara N (2001) Development of lentiviral vectors for antiangiogenic gene delivery. Cancer Gene Ther 8:879–889
Shifrin AL, Chirmule N, Gao GP, Wilson JM, Raper SE (2005) Innate immune responses to adenoviral vector-mediated acute pancreatitis. Pancreas 30:122–129

Shirakawa T (2009) Clinical trial design for adenoviral gene therapy products. Drug News Perspect 22:140–145

Siebzehnrubl FA, Vedam-Mai V, Azari H, Reynolds BA, Deleyrolle LP (2011) Isolation and characterization of adult neural stem cells. Method Mol Biol 750:61–77

Simonelli F, Maguire AM, Testa F, Pierce EA, Mingozzi F, Bennicelli JL, Rossi S, Marshall K, Banfi S, Surace EM, Sun J, Redmond TM, Zhu X, Shindler KS, Ying GS, Ziviello C, Acerra C, Wright JF, McDonnell JW, High KA, Bennett J, Auricchio A (2010) Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. Mol Ther 18:643–650

Sinnayah P, Lindley TE, Staber PD, Davidson BL, Cassell MD, Davisson RL (2004) Targeted viral delivery of Cre recombinase induces conditional gene deletion in cardiovascular circuits of the mouse brain. Physiol Genomics 18:25–32

Smith T, Idamakanti N, Kylefjord H, Rollence M, King L, Kaloss M, Kaleko M, Stevenson SC (2002) In vivo hepatic adenoviral gene delivery occurs independently of the coxsackievirus-adenovirus receptor. Mol Ther 5:770–779

Smith TA, Idamakanti N, Marshall-Neff J, Rollence ML, Wright P, Kaloss M, King L, Mech C, Dinges L, Iverson WO, Sherer AD, Markovits JE, Lyons RM, Kaleko M, Stevenson SC (2003a) Receptor interactions involved in adenoviral-mediated gene delivery after systemic administration in non-human primates. Hum Gene Ther 14:1595–1604

Smith TA, Idamakanti N, Rollence ML, Marshall-Neff J, Kim J, Mulgrew K, Nemerow GR, Kaleko M, Stevenson SC (2003b) Adenovirus serotype 5 fiber shaft influences in vivo gene transfer in mice. Hum Gene Ther 14:777–787

Sonpavde G, Thompson TC, Jain RK, Ayala GE, Kurosaka S, Edamura K, Tabata K, Ren C, Goltsov AA, Mims MP, Hayes TG, Ittmann MM, Wheeler TM, Gee A, Miles BJ, Kadmon D (2011) GLIPR1 tumor suppressor gene expressed by adenoviral vector as neoadjuvant intraprostatic injection for localized intermediate or high-risk prostate cancer preceding radical prostatectomy. Clin Cancer Res 17:7174–7182

Spear MA, Breakefield XO, Beltzer J, Schuback D, Weissleder R, Pardo FS, Ladner R (2001) Isolation, characterization, and recovery of small peptide phage display epitopes selected against viable malignant glioma cells. Cancer Gene Ther 8:506–511

Stein L, Roy K, Lei L, Kaushal S (2011) Clinical gene therapy for the treatment of RPE65-associated Leber congenital amaurosis. Expert Opin Biol Ther 11:429–439

Steiner B, Wolf S, Kempermann G (2006) Adult neurogenesis and neurodegenerative disease. Regen Med 1:15–28

Su JL, Lai KP, Chen CA, Yang CY, Chen PS, Chang CC, Chou CH, Hu CL, Kuo ML, Hsieh CY, Wei LH (2005) A novel peptide specifically binding to interleukin-6 receptor (gp80) inhibits angiogenesis and tumor growth. Cancer Res 65:4827–4835

Tamm I, Trepel M, Cardo-Vila M, Sun Y, Welsh K, Cabezas E, Swatterthwait A, Arap W, Reed JC, Pasqualini R (2003) Peptides targeting caspase inhibitors. J Biol Chem 278:14401–14405

Tani J, Faustine, Sufian JT (2011) Updates on current advances in gene therapy. West Ind Med J 60:188–194

Thomas CE, Edwards P, Wickham TJ, Castro MG, Lowenstein PR (2002) Adenovirus binding to the coxsackievirus and adenovirus receptor or integrins is not required to elicit brain inflammation but is necessary to transduce specific neural cell types. J Virol 76:3452–3460

Thomas CE, Ehrhardt A, Kay MA (2003) Progress and problems with the use of viral vectors for gene therapy. Nat Rev Genet 4:346–358

Touchefeu Y, Vassaux G, Harrington KJ (2011) Oncolytic viruses in radiation oncology. Radiother Oncol 99:262–270

Triozzi PL, Borden EC (2011) VB-111 for cancer. Expert Opin Biol Ther 11:1669–1676

Trono D (2000) Lentiviral vectors: turning a deadly foe into a therapeutic agent. Gene Ther 7:20–23

van Beusechem VW, Grill J, Mastenbroek DC, Wickham TJ, Roelvink PW, Haisma HJ, Lamfers ML, Dirven CM, Pinedo HM, Gerritsen WR (2002) Efficient and selective gene transfer into primary human brain tumors by using single-chain antibody-targeted adenoviral vectors with native tropism abolished. J Virol 76:2753–2762

van Zeeburg HJ, van Beusechem VW, Huizenga A, Haisma HJ, Korokhov N, Gibbs S, Leemans CR, Brakenhoff RH (2010) Adenovirus retargeting to surface expressed antigens on oral mucosa. J Gene Med 12:365–376

Vigne E, Dedieu JF, Brie A, Gillardeaux A, Briot D, Benihoud K, Latta-Mahieu M, Saulnier P, Perricaudet M, Yeh P (2003) Genetic manipulations of adenovirus type 5 fiber resulting in liver tropism attenuation. Gene Ther 10:153–162

Viru L, Heller G, Lehto T, Parn K, El Andaloussi S, Langel U, Merits A (2011) Novel viral vectors utilizing intron splice-switching to activate genome rescue, expression and replication in targeted cells. Virol J 8:243

Volpers C, Kochanek S (2004) Adenoviral vectors for gene transfer and therapy. J Gene Med Suppl 1:S164–S171

Von Seggern DJ, Chiu CY, Fleck SK, Stewart PL, Nemerow GR (1999) A helper-independent adenovirus vector with E1, E3, and fiber deleted: structure and infectivity of fiberless particles. J Virol 73:1601–1608

Vroemen M, Weidner N, Blesch A (2005) Loss of gene expression in lentivirus- and retrovirus-transduced neural progenitor cells is correlated to migration and differentiation in the adult spinal cord. Exp Neurol 195:127–139

Waehler R, Russell SJ, Curiel DT (2007) Engineering targeted viral vectors for gene therapy. Nat Rev Genet 8:573–587

Watkins SJ, Mesyanzhinov VV, Kurochkina LP, Hawkins RE (1997) The 'adenobody' approach to viral targeting: specific and enhanced adenoviral gene delivery. Gene Ther 4:1004–1012

Wennier S, Li S, McFadden G (2011) Oncolytic virotherapy for pancreatic cancer. Expert Rev Mol Med 13:e18

White SJ, Nicklin SA, Sawamura T, Baker AH (2001) Identification of peptides that target the endothelial cell-specific LOX-1 receptor. Hypertension 37:449–455

Wickham TJ (2000) Targeting adenovirus. Gene Ther 7:110–114

Wickham TJ (2003) Ligand-directed targeting of genes to the site of disease. Nat Med 9:135–139

Wickham TJ, Mathias P, Cheresh DA, Nemerow GR (1993) Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 73:309–319

Wickham TJ, Carrion ME, Kovesdi I (1995) Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs. Gene Ther 2:750–756

Wickham TJ, Roelvink PW, Brough DE, Kovesdi I (1996) Adenovirus targeted to heparan-containing receptors increases its gene delivery efficiency to multiple cell types. Nat Biotechnol 14:1570–1573

Wickham TJ, Tzeng E, Shears LL 2nd, Roelvink PW, Li Y, Lee GM, Brough DE, Lizonova A, Kovesdi I (1997) Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. J Virol 71:8221–8229

Witlox MA, Lamfers ML, Wuisman PI, Curiel DT, Siegal GP (2007) Evolving gene therapy approaches for osteosarcoma using viral vectors: review. Bone 40:797–812

Wonganan P, Croyle MA (2010) PEGylated adenoviruses: from mice to monkeys. Viruses 2:468–502

Wonganan P, Clemens CC, Brasky K, Pastore L, Croyle MA (2011) Species differences in the pharmacology and toxicology of PEGylated helper-dependent adenovirus. Mol Pharm 8:78–92

Xiong Z, Cheng Z, Zhang X, Patel M, Wu JC, Gambhir SS, Chen X (2006) Imaging chemically modified adenovirus for targeting tumors expressing integrin alphavbeta3 in living mice with mutant herpes simplex virus type 1 thymidine kinase PET reporter gene. J Nucl Med 47:130–139

Yao X, Yoshioka Y, Morishige T, Eto Y, Watanabe H, Okada Y, Mizuguchi H, Mukai Y, Okada N, Nakagawa S (2009) Systemic administration of a PEGylated adenovirus vector with a cancer-specific promoter is effective in a mouse model of metastasis. Gene Ther 16:1395–1404

Yao XL, Nakagawa S, Gao JQ (2011) Current targeting strategies for adenovirus vectors in cancer gene therapy. Curr Cancer Drug Target 11:810–825

Yoo GH, Moon J, Leblanc M, Lonardo F, Urba S, Kim H, Hanna E, Tsue T, Valentino J, Ensley J, Wolf G (2009) A phase 2 trial of surgery with perioperative INGN 201 (Ad5CMV-p53) gene therapy followed by chemoradiotherapy for advanced, resectable squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, and larynx: report of the Southwest Oncology Group. Arch Otolaryngol Head Neck Surg 135:869–874

Zhang X, Godbey WT (2006) Viral vectors for gene delivery in tissue engineering. Adv Drug Deliv Rev 58:515–534

Chapter 20
Regenerative Chimerism Bioengineered Through Stem Cell Reprogramming

Timothy J. Nelson, Almudena Martinez-Fernandez, Satsuki Yamada, and Andre Terzic

Abstract Regenerative medicine aims to restore damaged tissues in order to reverse disease progression and provide a sustainable solution that cures the root cause of the disease process. Although natural mechanisms of repair are ubiquitous, disruption of the homeostatic balance affects the equilibrium between health and disease due to insufficient tissue renewal in chronic degenerative conditions. Augmentation of the diseased tissue repair capacity through chimerism offers a strategy that spans all fields of medicine and surgery from natural chimerism for tissue rejuvenation, to surgical chimerism for organ replacement, to bioengineered chimerism for targeted regeneration. Technological breakthroughs in nuclear reprogramming now provide a platform to advance a broad range of solutions for regenerative medicine built on the foundation of pluripotent autologous stem cells. By optimizing the safety and effectiveness for stem cell production and ensuring tissue-specific differentiation of progenitors, induced pluripotent stem cells (iPS) offer an unprecedented opportunity to accelerate personalized applications with cell-based products to bioengineer health from disease.

20.1 Introduction: Regenerative Medicine

Regenerative medicine is primed by recent progress made in transplant medicine, stem cell biology and biomedical engineering to expand the therapeutic armamentarium available for the future of clinical practice. By providing patients with

T.J. Nelson, M.D., Ph.D. (✉) • A. Martinez-Fernandez, PharmD, Ph.D.
S. Yamada, M.D., Ph.D. • A. Terzic, M.D., Ph.D. (✉)
Marriott Heart Disease Research Program, Division of Cardiovascular Diseases, Departments of Medicine, Molecular Pharmacology and Experimental Therapeutics, and Medical Genetics, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA
e-mail: nelson.timothy@mayo.edu; terzic.andre@mayo.edu

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_20,

tissue-based products or biologics, regenerative medicine aims to ameliorate disease outcome while reducing the dependency on long-term palliative options. Advances in the science of regenerative medicine offer a transformative paradigm with curative objectives to address patient management demands unmet by non-curative approaches. Notably, the magnitude of chronic diseases increasingly challenges the sustainability of global health care systems (Cortese 2007; Waldman and Terzic 2007). In part, the success of modern medical care has allowed patients to survive initial presentation of acute disease processes, such as the pandemic of myocardial infarction, giving way to a prolonged course of disease management that relies primarily on palliative strategies to mitigate overt symptoms. Furthermore, the aging population is increasingly susceptible to degenerative diseases, which additionally escalates the growing burden on the health care system (Jahangir et al. 2007). Thus, the scope of chronic degenerative diseases will require interventions targeted towards the root cause of disease typically linked to progressive cellular destruction and irreversible loss of tissue function.

The concept of therapeutic repair (Fig. 20.1) encompasses the converging triad of *rejuvenation*, *replacement*, *and regeneration* as an overall goal to provide a sustainable cure (Nelson et al. 2008a). Although frequently underappreciated, rejuvenation provides the basis for self-healing from simple injuries of the skin to complex disease within tissues of the heart, liver, kidney, or brain. The unreliability of innate healing and the limited ability to augment inherent stem cell pools required to promote tissue homeostasis defines the unique challenges and opportunities of regenerative medicine. Likewise, transplant medicine exploits a replacement strategy as a valuable option to recycle used parts, and restore failing organ function by means of exogenous substitutes – it is however limited by donor shortage. Ultimately, stem cell-based regeneration has revealed the next frontier of medical therapy through delivery of essentially unlimited pools of autologous or allogeneic, naive or modified, natural or bioengineered progenitor cells to achieve structural and functional repair of all lineages. Collectively, stem cell-based regenerative medicine designed to supplement natural progenitors and facilitate chimeric healing of damaged tissues is poised to drive the evolution of medical sciences from traditional palliation towards curative therapy (Rosenthal 2003; Daley and Scadden 2008).

20.1.1 Rejuvenation

The rejuvenation strategy refers to self-renewal of tissues from endogenous stem cells within the individual body to promote tissue healing. This innate process of tissue refreshing enables the body to heal itself with younger cells through *de novo* biogenesis (Surani and McLaren 2006). Daughter cells can also be derived from reactivation of the cell cycle within mature cell types in response to stress or injury. This strategy replenishes tissue structure with endogenous stem cells, which generates natural chimeric tissue composed of cells distinguishable only by their birth dates. Although rejuvenation ensures continuous production of renewable

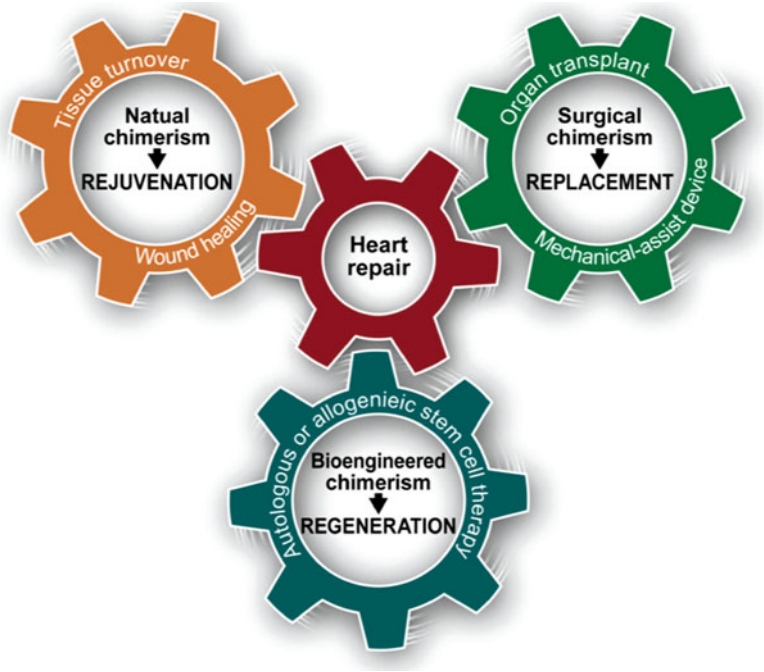


Fig. 20.1 Heart repair is the central goal of cardiovascular regenerative medicine that encompasses the strategic triad: rejuvenation, replacement, and regeneration illustrated here in the context of heart repair. Rejuvenation is defined as the repair of damaged tissue through activation of endogenous mechanisms that can stimulate natural tissue turnover and wound healing in order to replenish tissue function through "natural chimerism". Replacement is defined as repair of damaged tissue by recycling used parts through cardiac organ transplantation and now includes mechanical assist devices to achieve "surgical chimerism". Regeneration is defined as repair of damaged tissue through application of stem cells to generate new tissue and restore function following autologous or allogeneic cell-based "bioengineered chimerism". Collectively, these therapeutic strategies are recognized as an integrated approach for heart repair and could be applicable to damaged tissues and organs throughout the body

tissue required for long-term stress tolerance, most tissues are only partially able to self-renew. Moreover in the context of massive acute injury, such as myocardial infarction, inherent repair strategies are frequently inadequate (Anversa and Nadal-Ginard 2002). A boost in healing processes is likely required to stimulate adaptive response and promote adequate biogenesis of functional tissue to abrogate the progression of chronic heart diseases.

20.1.2 Replacement

The replacement strategy refers to transplantation of a donor tissue/organ that maintains functional integrity and re-establishes homeostasis within the host (Atala

2008). The field of surgery pioneered the concept of total replacement with the advent of solid organ transplantation. If the heart was irreversibly damaged, then replacing the diseased tissue with a functioning donor organ has remained a viable option. Cell-based replacement is also exemplified with routine use of donated red blood cells to replace circulating blood volume and treat life-threatening blood loss or anemia. This strategy recycles used cells, tissues, or organs to restore physiologic function for the recipient of the transplant upon establishment of a surgical chimera between donor tissue and host environment. A significant limitation of the replacement strategy remains the shortage of appropriate donors, and the difficulty to match the immunological criteria for safe and effective clinical applications.

20.1.3 Regeneration

The regenerative strategy refers to engraftment of progenitor cells that require *in vivo* growth and differentiation to establish repair within the host environment. Advances in hematology gave rise to the concept of regenerative cytotherapy with the identification of bone marrow-derived stem cells that could be harvested and transplanted in small quantities in order to reconstitute the entire hematopoietic stem cell pool naturally residing within the bone marrow (Kørbling and Estrov 2003). Success of stem cell transplantation was facilitated by engraftment into host bone marrow, which provides a protective environment to nurture the long-term survival of self-renewing stem cell properties. This strategy replenishes functional progenitor cells to allow on-demand differentiation of all hematopoietic lineages and sustained production of bioengineered chimeric tissue from donor stem cells within the host environment. Tissue-specific, non-hematopoietic stem cells have, furthermore, the capacity to re-establish lost function when ectopically transplanted into a wide range of diseased tissues as evident in diabetes, heart disease, and degenerative neurological conditions expanding regenerative applications.

20.2 Natural Chimerism: Heart Rejuvenation

Endogenous stem cells and self-repair mechanisms have been increasingly recognized as a natural process for tissue homeostasis (Laird et al. 2008). Fundamental to cardiac tissue rejuvenation is cardiomyocyte renewal through recruitment of endogenous progenitor pools within the body (Anversa et al. 2006; Torella et al. 2006). Notably, stem cell contribution to postnatal heart formation has been validated by the self/non-self chimerism characteristic of patients following allogeneic transplantation (Quaini et al. 2002; Kajstura et al. 2008a; Deb et al. 2003). Furthermore, innate stem cell loads increase in failing hearts and contribute to a regenerative response, involving ongoing derivation of cardiomyocytes from circulating or resident

progenitors (Kubo et al. 2008; Rupp et al. 2008). However, in the context of large-scale destruction following ischemic injury, the regenerative response required for tissue homeostasis is limited in its ability to salvage a deteriorating myocardium (Urbanek et al. 2005).

The magnitude of the natural process of cardiac tissue self-renewal is likely dependent on multiple factors such as patient age, disease status, co-morbidities, patient-specific medications, as well as genetic predispositions, epigenetics or ecogenetic influences. Utilizing quantification of radio-isotopes, introduced at high levels into the atmosphere during above ground nuclear bomb testing between the years 1955 and 1963 leading to subsequent DNA incorporation within living material, the birth date of individual cardiomyocytes was recently calculated (Bergmann et al. 2009). Based on these data, it has been estimated that cardiomyocytes can renew at <1% annually to achieve on average a renewal approaching up to 50% of the total heart mass over a lifespan (Bergmann et al. 2009). Although the magnitude is generally thought to be insufficient to compensate for severe tissue loss in acute disease states, the natural chimerism that is produced as a result of rejuvenation may gradually contribute to the prevention of heart disease and provide a significant protective mechanism of self-renewal to the heart, as originally suggested in transplant patients (Anversa et al. 2006; Torella et al. 2006). The precise mechanism of autologous self-renewal remains only partially addressed, but mounting evidence confirms the presence of *in situ* cardiogenic differentiation (Kajstura et al. 2008b; Hsieh et al. 2007). Direct evidence of allogeneic circulating stem cell contribution to the heart has been demonstrated in multiple patient-derived samples (Anversa et al. 2006; Torella et al. 2006; Quaini et al. 2002; Kajstura et al. 2008a). Importantly, this data from chromosomal mismatch does not preclude an active participation of resident stem cells in cardiac tissue renewal as calculated by the spectrum of de-identified cardiomyocyte birth dates. Therefore, based on the established paradigm of heart rejuvenation it is appropriate to surmise that augmentation of natural chimerism, either by reactivation of endogenous or transplantation of exogenous progenitor cells, offers a legitimate target to ameliorate the burden of chronic, degenerative heart disease presented herein as a disease paradigm (Fig. 20.1).

20.3 Surgical Chimerism: Heart Replacement

In response to end-stage heart failure in which the heart was damaged beyond reasonable probability for recovery, cardiac transplantation was pioneered over the past century to engineer a therapeutic option. Pre-clinical breakthroughs originated from the innovative efforts of Alexis Carrel along with Charles Guthrie who together succeeded to transplant the first heterotopic canine heart in 1905 (Carrel and Guthrie 1905). Over the next 60 years, significant discoveries in cardiac transplant biology and surgical techniques laid the fundamental groundwork for clinical translation that resulted in the first successful human-to-human cardiac transplantation performed by Christiaan Barnard (Lower and Shumway 1960; Hardy et al. 1964;

Barnard 1967). The proceeding decade witnessed decreased enthusiasm for the experimental procedure with poor survival rates and inevitable post-procedural complication due to allogeneic immune status. However, with the introduction of effective immunosuppression in the early 1980s (Oyer et al. 1983), technical improvements accelerated clinical practice of cardiac transplantation towards not only a viable strategy but into the standard of care for end-stage heart failure (Fig. 20.1). Today, there are more then 2,500 heart transplants done annually in the United States (Hunt et al. 2009); however, the numbers have not changed in the past decade despite a focused effort to address the organ shortage (Taylor et al. 2008). This has presented a significant clinical challenge for the estimated 100,000 patients in the United States alone that would be a potential candidate for this lifesaving procedure (Rosamond et al. 2008).

Due to the magnitude of the unmet need, alternative strategies such as mechanical assist devices have gained significant attention throughout this period (Fig. 20.1). The use of mechanical circulatory assist devices in refractory heart failure has been investigated in clinical trials for more than a decade. Extracorporeal devices have been used for short-term circulatory support in selected patients who are expected to have a reversible pathology and a transient need. Of note, advances in mechanical assist technology have produced devices that now offer remarkable hemodynamic support and have been introduced for long-term support of patients even in the ambulatory setting (Goldstein et al. 1998). Success of this technology has led to the concept of not only "bridging" to transplant or recovery, but now is being offered in selected patients not eligible for transplant as a permanent or "destination" therapy. The Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure (REMATCH) trial enrolled 129 patients for a 2-year follow-up period that demonstrated a 23% survival in mechanical device treated versus 8% survival with medical therapy alone (Rose et al. 1999). These data established the therapeutic value of mechanical support devices in the treatment algorithm of end-stage heart disease, and justified a role for patients that have a 1-year survival of less than 50%. Improved outcomes are likely to be reported as patient selection is refined, surgical and post-surgical care evolves, and devices are re-engineered for long-term sustainability. However, these advancements do little to prevent the pandemic of candidate patients with refractory heart failure and only expands palliative approaches that are largely limited to symptomatic management of a progressive disease.

20.4 Bioengineered Chimerism: Heart Regeneration

20.4.1 Recapitulating De Novo Cardiogenesis

Pluripotent stem cells have demonstrated the ability to contribute to chimeric tissues and illustrate the potential for exogenous stem cells to augment structure and function of host cardiac tissue. Chimeric offspring can be bioengineered through multiple

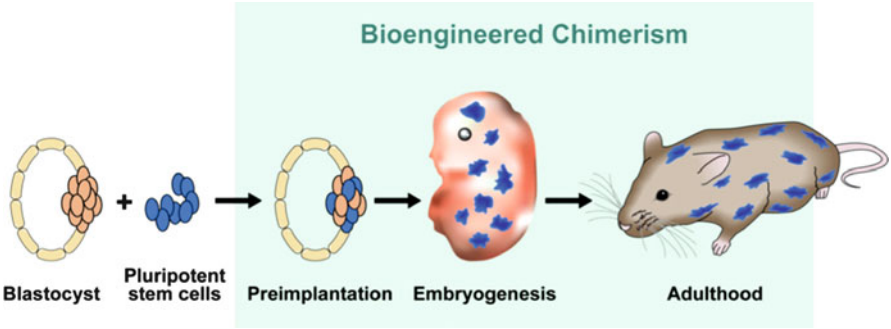


Fig. 20.2 Bioengineered chimerism. Adult chimeric offspring can be generated from pluripotent stem cells by manipulation of the blastocyst prior to implantation within surrogate mother. By injecting 10–20 cells into the cavity of a developing blastocyst or aggregation between compact morula and pluripotent stem cell, the preimplantation blastocyst becomes a random mixture of original blastomeres and transplanted stem cells. The chimeric inner cell mass gives rise to a mosaic pattern of chimeric tissue throughout development and are sufficient to produce adult offspring with random distributions of tissues derived from the transplanted pluripotent stem cells. The bioengineered chimerism offers unique opportunities to study the cell-autonomous defects of stem cells throughout development and into pathophysiological homeostasis of the adult system

techniques that place competent stem cells in direct contact with early stage embryos (Wood et al. 1993b; Wakayama et al. 2001; Nelson et al. 2009c; Stillwell et al. 2009). Two common methods of producing chimeric mice from pluripotent stem cells consist of either microinjection into the blastocoel cavity of a blastocyst-stage host embryo or by the non-coerced aggregation with a morula-stage host embryo (Wood et al. 1993a; Tam and Rossant 2003). Aggregation techniques include diploid aggregation in which host embryo is an 8-cell wild-type morula (Fig. 20.2) and tetraploid aggregation in which host embryo is the product of electrofusion of a two-cell embryo into a single cell containing two copies of the genome. In both diploid aggregation and blastocoel injections, the resulting fetus will be comprised of a mixture of stem cell-derived progeny along with lineages originating from the host embryo. Because host embryo has unaltered differentiation capacity, the transplanted pluripotent stem cells offer an equivalent source for the differentiating embryo and allow stochastic integration of the chimeric offspring. In contrast to the non-competitive combination of stem cells with similar differentiation capacity, tetraploid aggregation utilizes a partially defective host embryo and requires the transplanted stem cells to compensate for developmental deficiencies inherent within the host embryo (Nagy et al. 1993). In this way, bioengineering has confirmed the ability of chimeric tissues to rescue genetic defects and provide viable offspring from otherwise lethal mutations innate to the host environment. Case in point, the deletion of vascular endothelial growth factor (VEGF) disrupts the vascular development in the placenta and results in a developmental arrest (Carmeliet et al. 1996). However, chimeric complementation with pluripotent stem cells containing wild-type VEGF rescues the definitive pre-natal defects and produces viable offspring (Hirashima et al. 2003). Thus, chimeric tissue has provided a powerful platform for discovery sciences and establishes the potential therapeutic value of bioengineered chimerism.

Beyond cell-autonomous rescue of defective tissues, chimeric tissue reconstruction has also been demonstrated to repair disease-causing mutations through neomorphic (non-cell autonomous or paracrine) mechanisms (Fraidenraich et al. 2004). Tissue formation is dependent on multiple cell types that develop together and rely on the cooperative microenvironment, exemplified in cardiogenesis in which juxtaposition of definitive endoderm to secrete growth factors guides pre-cardiac mesoderm maturation (Lough and Sugi 2000; Foley et al. 2006). Therefore, mapping of defective signaling pathways in individual cardiac disease conditions that depend on paracrine support has provided therapeutic targets for chimeric tissue reconstruction. The translational value of this approach was originally discovered according to dramatic rescue of the embryonic lethal phenotype in Id1/Id3 knockout embryo upon blastocoel injection of wild-type embryonic stem cells (Fraidenraich et al. 2004). Repair of cardiogenesis in the defective embryos has been recapitulated with both indirect transplantation of embryonic stem cells into the peritoneal cavity of mother and the acellular delivery of insulin-like growth factor (IGF-1) as the principle active ingredient (Fraidenraich et al. 2004).

Extending the rational basis of chimeric tissue reconstruction for disease management, preemptive cell-based intervention has more recently been demonstrated for ischemic heart disease (Yamada et al. 2009). Embryonic stem cells delivered into an early stage host embryo were tested to determine whether bioengineered chimeric tissue could impact the tolerance of the adult to ischemic injury. Chimera offspring were generated through microinjection of pluripotent stem cells into pre-implantation embryos, and then examined for cardiac stress tolerance in adulthood. Indeed, bioengineered chimera demonstrated a functional and structural benefit compared to non-chimeric counterparts in the setting of coronary artery occlusion. The proof-of-concept provided the initial evidence of preventive regenerative medicine in the setting of myocardial infarction implemented through prenatal intervention (Yamada et al. 2009). Thus, chimeric incorporation of healthy progenitor cells into host embryos, before and after disease onset, has provided mechanistic insight to a wide range of pathology and increasingly offers a novel therapeutic strategy (Schneider et al. 2009).

20.4.2 Advancing Stem Cell-Based Cardiac Repair

Along with the paradigm shift that the heart is a self-healing organ came the design and implementation of clinical trials to test the hypothesis that additional stem cell load would lead to accelerated heart repair following ischemic injury (Leri et al. 2008). Promising pre-clinical data documenting improvement in cardiac performance following stem cell transplantation provided the foundation to test in patients the safety and feasibility of stem cell therapy in cardiac disease (Dimmeler et al. 2005; Segers and Lee 2008).

Autologous skeletal myoblasts were the initial cell type used in clinical trials (Menasché et al. 2001). Approximately 9×10^8 myoblasts were obtained from muscle biopsy and transplanted into the myocardium during open-heart surgery. Ventricular

tachycardia was recognized as a possible side-effect of therapy. However, in individual cases patients reported improvement in symptoms significant enough to decrease their heart failure class score, and had improvement in left ventricular ejection fraction. Subsequent trials have used lower concentrations of myoblasts, and have demonstrated a lower incidence of ventricular arrhythmias (Opie and Dib 2006). Long-term follow-up demonstrated improvement in clinical status and a decrease in hospitalizations for heart failure, while the risk of arrhythmia was appropriately controlled with medical therapy and/or device implantation (Hagège et al. 2006). A placebo controlled, multicenter phase III clinical trial (MAGIC) demonstrated significant decrease in left ventricular diameter after 6 months indicative of improved remodeling in patients with heart failure following myoblast injection directly into the myocardium, despite no significant change in systolic function of the treated heart muscle (Menasché et al. 2008).

The TOPCARE-AMI trial was designed to test the safety and feasibility of stem cell transplantation after acute myocardial infarction using circulating or bone marrow-derived progenitor cells (Schächinger et al. 2004). Initial studies demonstrate a safe clinical profile without ventricular arrhythmia, thrombus formation, distal embolization or dissection of coronary artery throughout a 1-year follow-up period. Furthermore, serial MRI imaging of the left ventricle demonstrated improved ejection fraction of ~8% as early as 4 months, and up to 12 months after transplantation. Safety and feasibility was independently confirmed using bone marrow cells transplanted into patients with a large ST-elevation myocardial infarction (Sánchez et al. 2006). The first randomized clinical trial, BOOST, examined patients after having an ST-elevation myocardial infarction that involved successful treatment by percutaneous stent placement into a single coronary artery (Drexler et al. 2006). Five days after optimal management according to standard medical practice, patients were treated with autologous bone marrow cell therapy and demonstrated a 6.7% improvement in ejection fraction compared to <1% improvement in the medically managed control cohort at 6-months follow-up. However, the significance of left ventricular function improvement in stem cell treated patients was not sustained at 18-month follow-up. The ASTAMI trial limited the inclusion criteria to acute myocardial infarction involving the left anterior descending coronary artery and randomized patients to receive bone marrow mononuclear cells via coronary artery delivery (Lunde et al. 2007). After a 6-month follow-up period, no significant difference between groups was detected in global left ventricular function. This randomized open-labeled study involving 100 patients confirmed the low risk of cell transplantation without increased risk of thrombosis, re-stenosis, or arrhythmia, and importantly showed significant improvement in exercise time and heart rate responses (Schächinger et al. 2006). Two phase II randomized clinical trials consisting of 200 patients in a multi-institutional study, REPAIR-AMI (Janssens et al. 2006), and 67 patients in the STEMI study (Perin et al. 2004) not only randomized patients, but also performed placebo injections in the control group via a similar coronary artery catheter approach. The primary outcome of functional improvement as measured by ejection fraction was significantly increased by 5.5% with cell transplantation in the REPAIR-AMI study compared to 3.0% in placebo group at

4 months. In a sub-group analysis it was surmised that initial ejection fraction of less than 49% had a significant benefit to cellular transplantation when compared to patients with baseline ejection fraction of greater than 49%. The STEMI study demonstrated significant decrease in left ventricular infarct size as measured by MRI but was unable to demonstrate any significant increase in left ventricular function between the placebo control and cell transplantation groups. Moreover, transendocardial injection of autologous bone marrow mononuclear cells in patients with end-stage ischemic heart disease has also been demonstrated to produce a durable therapeutic effect and improve myocardial perfusion and exercise capacity (Perin et al. 2004).

To date, over 3,000 patients with ischemic heart disease have received stem cell therapy in a clinical trial setting worldwide (Bartunek et al. 2007). Meta-analysis collectively indicates the safety profile of stem cell-based therapy, with modest improvements in functional parameters and apparent benefit in structural remodeling (Abdel-Latif et al. 2007). Ongoing optimization of the most appropriate cell type, selection of patient populations amenable to cell-based therapy, timing of intervention, and route of administration are the areas of focus to determine the clinical utility of cell-based therapy in cardiovascular disease.

20.5 Induced Pluripotent Stem Cells: A Platform for Unlimited Cardiac Repair

Beyond natural sources of stem cells that are limited by availability, immune intolerance, and lineage specification, bioengineered stem cell platforms are rapidly being developed for regenerative medicine applications. Converted from parental somatic cell types, bioengineered stem cells have acquired the ability to give rise to all cell types of the adult body, previously only possible from embryonic stem cells. Furthermore, recent studies have demonstrated that chimeric tissue from bioengineered stem cells is able to produce significant functional and structural repair in disease models.

20.5.1 Principles of Nuclear Reprogramming

Bioengineered stem cells offer the ability to provide unlimited supply of progenitor cells at any time point for virtually all cell types and tissues of the adult body starting from ordinary self-derived tissues (Fig. 20.3). By exploiting epigenetics and the microenvironment of somatic nuclei, reprogramming platforms aim to reverse cell fate of common cell types that are readily available in order to achieve conversion of a mature cell type back to the embryonic ground state (Jaenisch and Young 2008). Advancement of bioengineered platforms was realized through the pioneering work of somatic cell nuclear transfer technology that demonstrated the efficacy of transacting

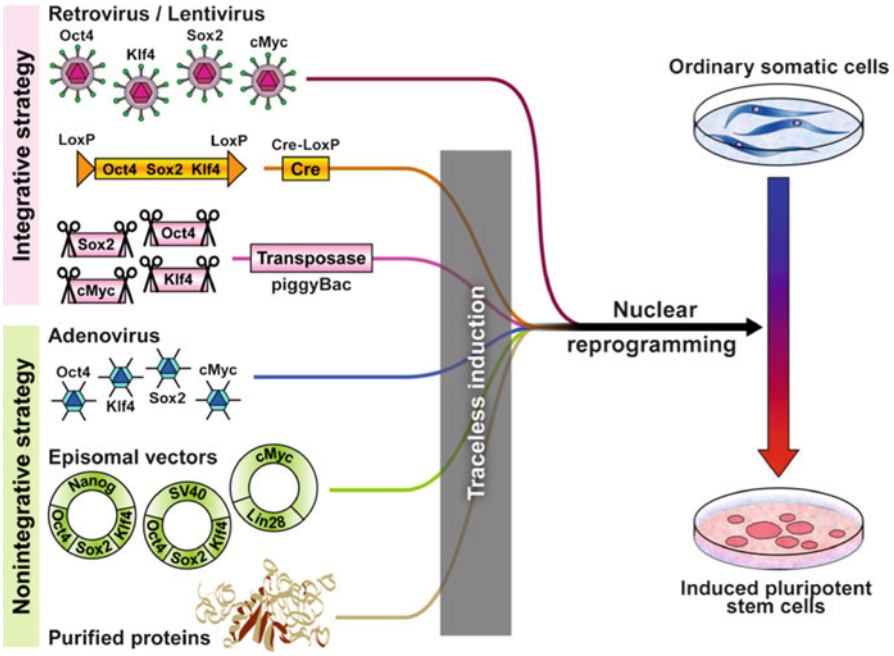


Fig. 20.3 Strategies for nuclear reprogramming of induced pluripotent stem cells. Ordinary somatic cell types can provide the parental source for nuclear reprogramming, including patient-specific tissue samples. Integrative strategies based on both retrovirus and lentivirus provided the initial successful model system to reprogram ordinary cells into iPS cells. The next-generation of this platform included the second step of removing the ectopic transgenes with either Cre recombinase or transposon-transposase systems, thus allowing a truly traceless approach. Alternatively, non-integrative strategies became feasible with both viral transduction of adenovirus and episomal vectors or plasmid-based constructs. These strategies delivered the same ectopic stemness-related factors without the risk of insertional mutagenesis, although at lower overall efficiency of nuclear reprogramming. Furthermore, non-integrative strategies also include protein-based bioengineering to deliver the transient levels of stemness-related factors needed for successful nuclear reprogramming

factors present within the mammalian oocytes, conserved across species, to reprogram somatic cell nuclei to an undifferentiated state (Yang et al. 2007; Beyhan et al. 2007). Thus, therapeutic cloning refers to somatic cell nuclear transfer (SCNT) in which the nuclear content of a somatic cell from an individual is transferred into an enucleated donor egg to derive blastocysts that contain pluripotent embryonic-like stem cells. In this way, SCNT has produced cloned embryonic stem cells from multiple mammalian somatic cell biopsies (Hall and Stojkovic 2006; Sha et al. 2009; Byrne et al. 2007). The pluripotency of derived cells has been confirmed through germline transmission and reproductive cloning. However, due to technological limitations, cloned human blastocytes have only recently been achieved albeit in low efficiency (French et al. 2008), and successful isolation of embryonic stem cells from the inner cell mass has yet to be demonstrated with human protocols.

Nuclear reprogramming of adult somatic cells through ectopic introduction of a small number of pluripotency-associated transcription factors is a streamline approach to induce an embryonic stem cell-like phenotype (Takahashi and Yamanaka 2006; Yamanaka 2008, 2009a, c). Transcription factors sets, Oct4, Sox2, c-Myc and Klf4 or alternatively Oct4, Sox2, Nanog and Lin28 (Yu et al. 2007), are sufficient to reprogram human somatic cells by inducing a sequential reversal into a pluripotent phenotype (Fig. 20.3). The process of nuclear reprogramming requires controlled expression of specific stemness factors in the proper stoichiometry for a defined period of time (Meissner et al. 2007; Maherali et al. 2007; Takahashi et al. 2007b; Yamanaka 2007; Park et al. 2008a, b; Papapetrou et al. 2009). Multiple source tissue has been successfully reprogrammed such as fibroblasts (Takahashi et al. 2007a), keratinocytes (Aasen et al. 2008), blood (Loh et al. 2009), or adipose tissue (Sun et al. 2009). The balanced exposure of ectopic factors is sufficient to induce telomere elongation (Marion et al. 2009b), histone modifications (Deng et al. 2009), secondary gene expression profiles (Mikkelsen et al. 2008), and cellular metamorphosis that collectively re-establish a self-stabilizing phenotype of pluripotency (Silva et al. 2009). Reprogramming occurs typically within weeks of coerced equilibrium of the trans-acting factors that can be delivered to the nucleus either by plasmids, viruses, or recombinant proteins (Fig. 20.3). Thereby, ectopic transgene expression initiates a sequence of stochastic events that eventually transforms a small fraction of cells (<0.5%) to acquire this imposed pluripotent state characterized by a stable epigenetic environment indistinguishable from the blastocyst–derived natural stem cell *milieu*. The acquired pluripotent ground state culminates in the maintenance of the unique developmental potential to differentiate into all germ layers. In this way, induced pluripotent stem cells (iPS) with the ability to derive patient specific progenitor cells should largely eliminate the concern of stem cell shortage, immune rejection of non-autologous sources, and inadequate capacity for lineage specification (Nishikawa et al. 2008; Nakagawa et al. 2008; Park et al. 2008c). Moreover, iPS based technology will facilitate the production of cell line panels that closely reflect the genetic diversity of a population enabling the discovery, development and validation of diagnostics and therapeutics tailored for each individual (Waldman and Terzic 2008).

20.5.2 Autologous Pluripotent Stem Cells

Bioengineered platforms bypass the need for embryo extraction to generate true pluripotent stem cell phenotypes from autologous sources. In the mouse, bioengineering has yielded iPS clones sufficient for complete *de novo* embryogenesis as the highest evidence of pluripotent stringency (Zhao et al. 2009; Boland et al. 2009) and in humans, by giving rise to all three germ layers, has ensured comprehensive multi-lineage tissue differentiation. Self-derived iPS cells will be recognized within the transplanted hosts as native tissue due to their autologous status, but will also require new level of protection from dysregulated growth. The next generation of

bioengineered stem cells will likely include specialized properties to improve stress tolerance, streamline differentiation capacity, and increase engraftment/survival to improve regenerative potential.

20.5.2.1 First-Generation Technology

Retroviral and lentiviral approaches offered the initial methodology that launched the field, and established the technological basis of nuclear reprogramming with rapid confirmation across integrating vector systems (Takahashi and Yamanaka 2006; Yu et al. 2007; Meissner et al. 2007; Aasen et al. 2008; Okita et al. 2007; Aoi et al. 2008; Huangfu et al. 2008; Eminli et al. 2008; Kim et al. 2008; Hanna et al. 2008; Feng et al. 2009). The risk of oncogenic genes and insertional mutagenesis inherent to stable genomic integration has been recognized as potential limitations from the onset of this technology. However, distinct advantages of the retroviral-based vector systems enabled critical insight to the mechanisms of reprogramming. Retroviral and lentiviral systems have built-in sequences that silence the process of transcription upon pluripotent induction, thus persistent exposure to ectopic gene expression was temporally restricted at the time of re-induction of pluripotency. This allows an essential observation to be made in that successful self-maintenance of the pluripotent state was possible without long-term transgene expression. Thereby, systems were envisioned for transient production of stemness-related genes without integration into the genome to improve the safety and efficacy of nuclear reprogramming. The first proof-of-principle was achieved by non-integrating viral vector systems, such as adenovirus (Stadtfeld et al. 2008), and confirmed by repeated exposure to extra-chromosomal plasmid-based transgenes (Okita et al. 2008). Importantly, these reports demonstrated that expression of stemness-related factors was required for only a limited timeframe until progeny developed autonomous self-renewal, establishing nuclear reprogramming as a bioengineered process that resets a sustainable pluripotent cell fate independent of permanent genomic modifications. The inherent inefficiency of non-integrated technologies has however hindered broader applicability and stimulated the search for more efficient methodology.

20.5.2.2 Second-Generation Technology

The latest innovation that advances iPS-based technology towards clinical applications has most recently been reported in which non-viral approaches are capable of high-efficiency production (Kaji et al. 2009; Woltjen et al. 2009). These approaches are dependent on short sequences of mobile genetic elements that can be used to integrate transgenes into host cell genomes and provide a genetic tag to “cut and paste” flanked genomic DNA sequences (Nelson and Terzic 2009). The piggyBac (PB) system couples enzymatic cleavage with sequence specific recognition using a transposon/transposase interaction to ensure high efficiency removal of flanked

DNA without residual footprint. Importantly, this technology achieves a traceless transgenic approach in which non-native genomic sequences that are transiently required for nuclear reprogramming can be removed upon induction of pluripotency. Specifically, using the PB transposition system with randomly integrated stemness-related transgenes, recent studies have demonstrated that disposal of ectopic genes could be efficiently regulated upon induction of self-maintaining pluripotency according to expression of the transposase enzyme without infringement on genomic stability (Woltjen et al. 2009). This state-of-the-art system is qualified to allow safe integration and removal of ectopic transgenes, improving the efficiency of iPS production and facilitating a minimally invasive methodology without permanent modifications to the progeny. Alternatively, the security of unmodified genomic intervention can be achieved with non-integrating episomal vectors (Yu et al. 2009). Collectively, these recent strategies allow genetically unmodified progenitor cells to acquire the capacity of pluripotency.

Alternatively, high-stringency iPS cells have also been produced with proteins in the absence of genetic material (Zhou et al. 2009; Kim et al. 2009). The protein-only approach has successfully induced reprogramming with either whole cell extract enriched in four stemness factors used in combination with pharmacological induction of cell permeability or with stemness factors modified by cell-permeating polyarginine tag (Zhou et al. 2009). Although the reprogramming efficiency compared to genetic methodology is reduced, there are emerging strategies that complement the influence of stemness factors exposure within somatic cells. Namely, small molecules targeting histone modifications have increased reprogramming efficiencies (Shi et al. 2008) along with the latest discovery that tumor suppressor gene, p53, is responsible for inhibiting the reprogramming process (Banito et al. 2009; Hong et al. 2009; Utikal et al. 2009; Marion et al. 2009a; Li et al. 2009; Kawamura et al. 2009). Thereby, transient knockdown of p53 according to siRNA strategies targeting the breakdown of mRNA or overexpression of MDM-2 to increase p53 protein degradation has proven to successfully increase the overall efficiency 1–2 orders of magnitude with up to 20% of selected cells undergoing *bona fide* reprogramming (Banito et al. 2009; Hong et al. 2009; Utikal et al. 2009; Marion et al. 2009a; Li et al. 2009; Kawamura et al. 2009). Together, the rapid advancements in nuclear reprogramming have accelerated bioengineered pluripotent stem cells closer to the milestones required for possible clinical translation.

20.5.3 Regeneration of Diseased Tissues

To date, therapeutic benefit of iPS-based technology has been tested in four disease models, namely sickle cell anemia (Hanna et al. 2007), Parkinson's disease (Wernig et al. 2008), hemophilia A (Xu et al. 2009), and ischemic heart disease (Nelson et al. 2009d). As differentiation protocols are refined to produce "on-demand" tissue-specific progeny, additional pre-clinical disease models will be screened to address the full regenerative value of iPS technology.

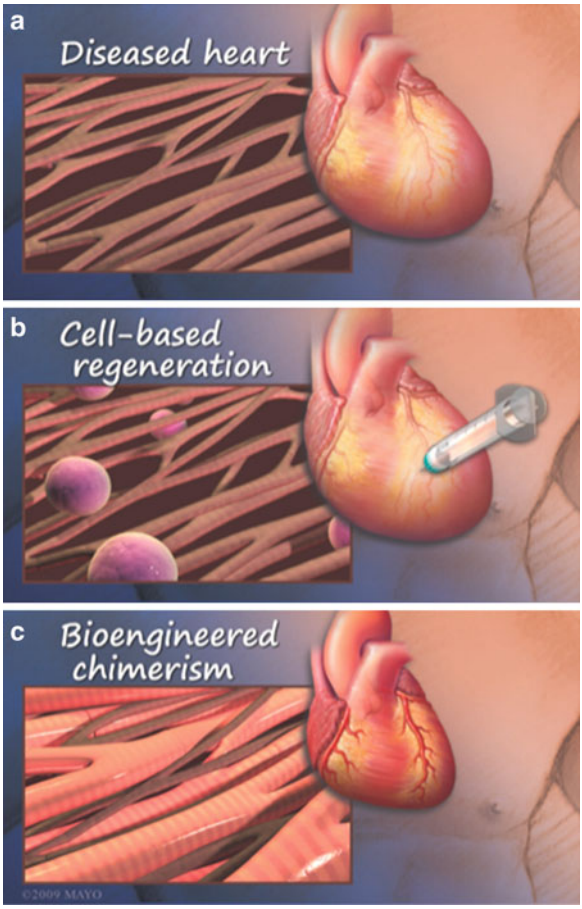


Fig. 20.4 Induced pluripotent stem cell-based repair of heart disease. (**a**) Patients with heart failure develop progressive disease that results in weakened and dilated heart muscle, unable to function normally. (**b**) Direct intramyocardial delivery of stem cells leads to iPS engraftment within diseased heart. (**c**) Bioengineered chimerism according to iPS-based therapy has demonstrated functional benefit to the diseased heart muscle with direct evidence for stable engraftment and *in vivo* cardiovascular regeneration of new tissue

In regards to cardiogenesis, embryonic stem cells have a spontaneous propensity for cardiac differentiation that fulfills an early requirement for heart formation during embryonic development. Compared to the gold standard of embryonic stem cells, iPS have demonstrated a similar capacity for *in vitro* cardiac differentiation. Using methodology established for embryonic stem cell-derived cardiogenesis, iPS differentiating in embryoid bodies or aggregates of tissue starting with 400–500 cells systematically produce mesoderm lineages and pre-cardiac cytotypes according to established gene expression profiles (Schenke-Layland et al. 2008). Within appropriate time frames, mouse and human tissue give rise to early cardiomyocytes with spontaneous beating activity (Fig. 20.4). This tissue expresses

contractile proteins, such as troponin and actinin. Furthermore, the cardiac-like tissue is regulated according to excitable inputs through gap junctions and calcium from extracellular and intracellular sources. As the cardiac tissue matures *in vitro*, specialized heart muscle cells become evident with assembly of characteristic ion channel sets responsible for physiological regulation of cardiac contraction and electrical conductance within ventricular, atrial, and pacemaker cell types (Schenke-Layland et al. 2008; Narazaki et al. 2008; Mauritz et al. 2008; Zhang et al. 2009; Yokoo et al. 2009).

The therapeutic value of iPS in cardiovascular medicine was recently documented in a model of acute myocardial infarction (Nelson et al. 2009d). Post-ischemic cardiac performance was compared in randomized cohorts transplanted with parental fibroblasts versus bioengineered iPS (Nelson et al. 2009d). As quantified by echocardiography, occlusion of anterior epicardial coronary blood flow permanently impaired regional wall motion and cardiac function. Treatment with parental fibroblasts was unable to improve performance of post-ischemic hearts (Fig. 20.4). Yet, iPS intervention in the acute stages of myocardial infarction improved cardiac contractility by 4 weeks post-transplantation. Functional benefit in response to iPS therapy was verified by the improvement in fractional shortening and regional septal wall thickness during contraction that demonstrate coordinated concentric contractions visualized by long-axis and short-axis 2-D imaging (Nelson et al. 2009d). Beyond functional deterioration, maladaptive remodeling with detrimental structural changes prognosticates poor outcome following ischemic injury to the heart. In contrast to non-reparative fibroblasts, iPS-based intervention attenuated global left ventricular diastolic diameter predictive of decompensated heart disease. A consequence of pathologic structural remodeling is evident by prolongation of the QT interval, which increases risk of life-threatening arrhythmias (Nelson et al. 2009d). Successful iPS treatment prevented structural remodeling to avoid deleterious effects on electrical conductivity.

These real-time surrogates for tissue remodeling have been confirmed by gross inspection of specimens. Autopsy allowed histological analysis to determine the extent of scar tissue formation within the post-ischemic region of the anterior circulation distal to the coronary ligation (Nelson et al. 2009d). In contrast to parental fibroblasts, iPS treatment halted structural deterioration with decreased fibrotic scarring and induction of remuscularization with *de novo* heart muscle tissue along with evidence for angiogenesis according to vascular endothelial markers. Surgical dissection verified absence of tumor infiltration or dysregulated cell expansion following iPS transplantation in the myocardium itself, as well as in organs with high metastatic risk such as the liver, lung and spleen. Collectively, iPS-derived regeneration of the ischemic heart has been demonstrated at multiple levels of stringency that include cellular, tissue, structural, functional, and metabolic levels, providing a foundation for development of this novel platform towards clinical applicability (Nelson et al. 2009d).

With ongoing understanding of principles of myocardial regeneration (Srinivas et al. 2009), clinical translation of iPS technology faces similar challenges that have in part been addressed by natural stem cell applications, including embryonic stem

cells approved early in 2009 by the Food and Drug Administration in the United States for trials involving patients with incurable spinal cord injuries. The first universal obstacle for clinical translation of pluripotent stem cell technology is unregulated tumor formation (Li et al. 2008). Even a limited contamination of undifferentiated cells can, in theory, result in the formation of dysregulated tumors. Therefore, a critical milestone is to secure differentiation of iPS into the required cell type, purifying them away from residual undifferentiated precursors prior to transplantation (Yamanaka 2009a; Li et al. 2008). This becomes a unique challenge for iPS technology when the immune system is no longer involved in the elimination process of dysregulated foreign tissue, active with embryonic stem cell applications. The second issue that is unique to iPS is the accuracy of complete reprogramming of ordinary cells into pluripotent progeny. Inadequate conversion according to nuclear reprogramming strategies could result in impaired differentiation of iPS cells into target tissues required for specific applications (Yamanaka 2009a). Third, the issue of persistent transgene expression in iPS progeny requires careful consideration. Generally, iPS cells have been produced by transduction of ordinary cells with retroviruses or lentiviruses carrying ectopic transgenes in order to efficiently transfer stable expression into the host nucleus. This creates the risk of not only continuous expression of transgenes that are known to promote dysregulated tumor growth, but also involves permanent genomic modifications that raise the concern for insertion mutagenesis of endogenous loci.

Cardiac tissue specificity from stem cells has been investigated for more than a decade and as of yet no single gene or cluster of genes has been identified to secure cardiac differentiation. However, recent studies have significantly enriched the cardiac propensity with either exogenous growth factors (Behfar et al. 2008), cell sorting of cardiac progenitors (Nelson et al. 2008c; Moretti et al. 2006; Kattman et al. 2006; Yang et al. 2008), or genetically engineering pre-cardiac pathways all to encourage cardiogenesis from primitive stem cell pools (Takeuchi and Bruneau 2009). Collectively, these technologies offer the rational basis to design strategies to ensure cardiogenic specification and avoidance of undifferentiated subpopulations prior to transplantation. The crucial balance between lineage specification and progenitor cell proliferation (Martinez-Fernandez et al. 2009) will be essential to develop a robust manufacturing process that can be scaled and applied to clinical grade production of a cardiac stem cell-based product.

In order to translate iPS technology into clinical reality for heart disease, additional milestones will need to be considered. First, the target patient population will need to be identified based on disease-severity and lack of alternative options to justify inclusion into a first-in-man study. Many patients are too severely deconditioned or have significant co-morbidities to allow consideration for heart transplant, thus limiting treatment strategies to palliative medicines and procedures. This category of patients needs to be considered a priority in terms of experimental cell-based interventions. An advantage with autologous iPS technology is that no toxic immunosuppression is required, yet provides a unique strategy to overcome poor natural stem cell pools in elderly patients, limiting more traditional regenerative approaches. Thus, iPS-based products should be considered in patients with no other options to

decrease not only symptoms but also the need for hospitalization along with expensive yet invasive palliative management strategies such as destination left ventricular assist devices. Next, a good-manufacturing-practice production process and facility will need to be developed and implemented to ensure clinical-grade production of patient-derived iPS cells, as well as tissue-specific differentiation for targeted applications. Finally, regulatory agencies will require evidence of proper engraftment, survival, and safety of transplanted iPS-derived progeny (Nelson et al. 2008b). This will require proof-of-principle studies using clinical grade cell products in disease model systems encompassing comparative effectiveness for optimized outcomes (Nelson et al. 2008b, 2009b).

20.6 Clinical Perspective

Built on emerging discoveries in stem cell biology (Nelson et al. 2008a), regenerative medicine has begun to define the scope of future clinical practice (Nelson et al. 2009b; Waldman et al. 2007). Regenerative medicine and stem cell biology cross all disciplines of medicine/surgery, and provide a universal paradigm of curative goals based on scientific discovery and clinical translation. The challenges to realize the full potential of stem cell biology remain substantial, and thus requires integration of multidisciplinary teams with expertise to form a dedicated regenerative medicine community of practice (Nelson et al. 2009a). Building on the foundation of transplant medicine, regenerative medicine will continue to expand and implement technologies to treat new diseases at earlier stages with safer and more effective outcomes, not achievable with current standards of care. Individualized treatment algorithms for regenerative medicine will require quantification of the inherent reparative potential to determine patients that would benefit from stem cell therapy in order to target personalized regenerative medicine solutions.

Induced nuclear reprogramming through ectopic transgene expression of stemness factors offers a revolutionary strategy for embryo-independent derivation of autologous pluripotent stem cells from an ordinary adult source (Yamanaka 2009b). In such, iPS have attained functions previously demonstrated only by natural embryonic stem cells to independently produce all tissues types and develop the complete organism within an embryonic environment. To date with regard to cardiovascular applications, the reprogrammed iPS progeny have established the therapeutic value for cardiac tissue regeneration in a setting of experimental ischemic heart disease (Nelson et al. 2009d). Specifically, transplantation of iPS in the acutely ischemic myocardium yielded structural and functional repair to secure performance recovery as qualified clones contributed to *in vivo* tissue reconstruction with "on-demand" cardiovasculogenesis. Moreover, Parkinson's disease, sickle cell anemia, and hemophilia A are early examples of successful iPS applications in disease models (Nelson et al. 2010). Furthermore, patient-specific iPS cells have been generated from individuals with diabetes, amyotrophic lateral sclerosis, Fanconi anemia, and myeloproliferative disorders (Yamanaka 2007; Park et al. 2008a; Maehr et al. 2009; Dimos

et al. 2008; Ye et al. 2009; Raya et al. 2009). Therefore, converting self-derived fibroblasts into reparative progenitors can now be considered as a goal of regenerative medicine to individualize treatment algorithms for multi-lineage repair. In this way, clinical-grade, pluripotent, autologous stem cells offer a unique bioengineered tool to repair disease tissue through chimeric integration.

References

Aasen T, Raya A, Barrero MJ et al (2008) Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol 26:1276–1284

Abdel-Latif A, Bolli R, Tleyjeh IM et al (2007) Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. Arch Intern Med 167:989–997

Anversa P, Nadal-Ginard B (2002) Myocyte renewal and ventricular remodelling. Nature 415:240–243

Anversa P, Kajstura J, Leri A et al (2006) Life and death of cardiac stem cells: a paradigm shift in cardiac biology. Circulation 113:1451–1463

Aoi T, Yae K, Nakagawa M et al (2008) Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science 321:699–702

Atala A (2008) Advances in tissue and organ replacement. Curr Stem Cell Res Ther 3:21–31

Banito A, Rashid ST, Acosta JC et al (2009) Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev 23:2134–2139

Barnard CN (1967) A human cardiac transplant: an interim report of a successful operation performed at Groote Schuur Hospital, Capetown. S Afr Med J 41:1271

Bartunek J, Vanderheyden M, Wijns W et al (2007) Bone-marrow-derived cells for cardiac stem cell therapy: safe or still under scrutiny? Nat Clin Pract Cardiovasc Med 4(Suppl 1):S100–S105

Behfar A, Faustino RS, Arrell DK et al (2008) Guided stem cell cardiopoiesis: discovery and translation. J Mol Cell Cardiol 45:523–529

Bergmann O, Bhardwaj RD, Bernard S et al (2009) Evidence for cardiomyocyte renewal in humans. Science 324:98–102

Beyhan Z, Iager AE, Cibelli JB (2007) Interspecies nuclear transfer: implications for embryonic stem cell biology. Cell Stem Cell 1:502–512

Boland MJ, Hazen JL, Nazor KL et al (2009) Adult mice generated from induced pluripotent stem cells. Nature 461:91–94

Byrne JA, Pedersen DA, Clepper LL et al (2007) Producing primate embryonic stem cells by somatic cell nuclear transfer. Nature 450:497–502

Carmeliet P, Ferreira V, Breier G et al (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439

Carrel A, Guthrie CC (1905) The transplantation of veins and organs. Am Med 10:1101

Cortese DA (2007) A vision of individualized medicine in the context of global health. Clin Pharmacol Ther 82:491–493

Daley GQ, Scadden DT (2008) Prospects for stem cell-based therapy. Cell 132:544–548

Deb A, Wang S, Skelding KA et al (2003) Bone marrow-derived cardiomyocytes are present in adult human heart: a study of gender-mismatched bone marrow transplantation patients. Circulation 107:1247–1249

Deng J, Shoemaker R, Xie B et al (2009) Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. Nat Biotechnol 27:353–360

Dimmeler S, Zeiher AM, Schneider MD (2005) Unchain my heart: the scientific foundations of cardiac repair. J Clin Invest 115:572–583

Dimos JT, Rodolfa KT, Niakan KK et al (2008) Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science 321:1218–1221

Drexler H, Meyer GP, Wollert KC (2006) Bone-marrow-derived cell transfer after ST-elevation myocardial infarction: lessons from the BOOST trial. Nat Clin Pract Cardiovasc Med 3(Suppl 1):S65–S68

Eminli S, Utikal J, Arnold K et al (2008) Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression. Stem Cells 26:2467–2474

Feng B, Jiang J, Kraus P et al (2009) Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. Nat Cell Biol 11:197–203

Foley AC, Gupta RW, Guzzo RM et al (2006) Embryonic heart induction. Ann NY Acad Sci 1080:85–96

Fraidenraich D, Stillwell E, Romero E et al (2004) Rescue of cardiac defects in id knockout embryos by injection of embryonic stem cells. Science 306:247–252

French AJ, Adams CA, Anderson LS et al (2008) Development of human cloned blastocysts following somatic cell nuclear transfer with adult fibroblasts. Stem Cells 26:485–493

Goldstein DJ, Oz MC, Rose EA (1998) Implantable left ventricular assist devices. N Engl J Med 339:1522–1533

Hagège AA, Marolleau JP, Vilquin JT et al (2006) Skeletal myoblast transplantation in ischemic heart failure: long-term follow-up of the first phase I cohort of patients. Circulation 114(1 Suppl):I108–I113

Hall VJ, Stojkovic M (2006) The status of human nuclear transfer. Stem Cell Rev 2:301–308

Hanna J, Wernig M, Markoulaki S et al (2007) Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science 318:1920–1923

Hanna J, Markoulaki S, Schorderet P et al (2008) Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell 133:250–264

Hardy JD, Chavez CM, Kurrus FD et al (1964) Heart transplantation in man. JAMA 188:114

Hirashima M, Lu Y, Byers L et al (2003) Trophoblast expression of fms-like tyrosine kinase 1 is not required for the establishment of the maternal-fetal interface in the mouse placenta. Proc Natl Acad Sci USA 100:15637–15642

Hong H, Takahashi K, Ichisaka T et al (2009) Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature 460:1132–1135

Hsieh PC, Segers VF, Davis ME et al (2007) Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nat Med 13:970–974

Huangfu D, Osafune K, Maehr R et al (2008) Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol 26:1269–1275

Hunt SA, Abraham WT, Chin MH et al (2009) 2009 focused update incorporated into the ACC/AHA 2005 guidelines for the diagnosis and management of heart failure in adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines: developed in collaboration with the International Society for Heart and Lung Transplantation. Circulation 119:e391–e479

Jaenisch R, Young R (2008) Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell 132:567–582

Jahangir A, Sagar S, Terzic A (2007) Aging and cardioprotection. J Appl Physiol 103:2120–2128

Janssens S, Dubois C, Bogaert J et al (2006) Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. Lancet 367:113–121

Kaji K, Norrby K, Paca A et al (2009) Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature 458:771–775

Kajstura J, Hosoda T, Bearzi C et al (2008a) The human heart: a self-renewing organ. Clin Transl Sci 1:80–86

Kajstura J, Urbanek K, Rota M et al (2008b) Cardiac stem cells and myocardial disease. J Mol Cell Cardiol 45:505–513

Kattman SJ, Huber TL, Keller GM (2006) Multipotent flk-1^{+} cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. Dev Cell 11:723–732

Kawamura T, Suzuki J, Wang YV et al (2009) Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature 460:1140–1144
Kim JB, Zaehres H, Wu G et al (2008) Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 454:646–650
Kim D, Kim CH, Moon J et al (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell 6:472–476
Kørbling M, Estrov Z (2003) Adult stem cells for tissue repair – a new therapeutic concept? N Engl J Med 349:570–582
Kubo H, Jaleel N, Kumarapeli A et al (2008) Increased cardiac myocyte progenitors in failing human hearts. Circulation 118:649–657
Laird DJ, von Andrian UH, Wagers AJ (2008) Stem cell trafficking in tissue development, growth, and disease. Cell 132:612–630
Leri A, Kajstura J, Anversa P et al (2008) Myocardial regeneration and stem cell repair. Curr Probl Cardiol 33:91–153
Li JY, Christophersen NS, Hall V et al (2008) Critical issues of clinical human embryonic stem cell therapy for brain repair. Trends Neurosci 31:146–153
Li H, Collado M, Villasante A et al (2009) The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature 460:1136–1139
Loh Y, Agarwal S, Park I et al (2009) Generation of induced pluripotent stem cells from human blood. Blood 113:5476–5479
Lough J, Sugi Y (2000) Endoderm and heart development. Dev Dyn 217:327–342
Lower RR, Shumway NE (1960) Studies on the orthotopic homotransplantation of the canine heart. Surg Forum 11:18
Lunde K, Solheim S, Aakhus S (2007) Exercise capacity and quality of life after intracoronary injection of autologous mononuclear bone marrow cells in acute myocardial infarction: results from the Autologous Stem cell Transplantation in Acute Myocardial Infarction (ASTAMI) randomized controlled trial. Am Heart J 154:710.e1-8
Maehr R, Chen S, Snitow M et al (2009) Generation of pluripotent stem cells from patients with type 1 diabetes. Proc Natl Acad Sci USA 106:15768–15773
Maherali N, Sridharan R, Xie W et al (2007) Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell 1:55–70
Marion RM, Strati K, Li H et al (2009a) A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. Nature 460:1149–1153
Marion RM, Strati K, Li H et al (2009b) Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells. Cell Stem Cell 4:141–154
Martinez-Fernandez A, Nelson TJ, Yamada S et al (2009) iPS programmed without c-MYC yield proficient cardiogenesis for functional heart chimerism. Circ Res 105:648–656
Mauritz C, Schwanke K, Reppel M et al (2008) Generation of functional murine cardiac myocytes from induced pluripotent stem cells. Circulation 118:507–517
Meissner A, Wernig M, Jaenisch R (2007) Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat Biotechnol 25:1177–1181
Menasché P, Hagège AA, Scorsin M et al (2001) Myoblast transplantation for heart failure. Lancet 357:279–280
Menasché P, Alfieri O, Janssens S et al (2008) The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. Circulation 117:1189–1200
Mikkelsen TS, Hanna J, Zhang X et al (2008) Dissecting direct reprogramming through integrative genomic analysis. Nature 454:49–55
Moretti A, Caron L, Nakano A et al (2006) Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 127:1151–1165
Nagy A, Rossant J, Nagy R et al (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci USA 90:8424–8428
Nakagawa M, Koyanagi M, Tanabe K et al (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 26:101–106

Narazaki G, Uosaki H, Teranishi M et al (2008) Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. Circulation 118:498–506
Nelson TJ, Terzic A (2009) Induced pluripotent stem cells: reprogrammed without a trace. Regen Med 4:333–355
Nelson TJ, Behfar A, Terzic A (2008a) Strategies for therapeutic repair: the "R3" regenerative medicine paradigm. Clin Transl Sci 1:168–171
Nelson TJ, Behfar A, Terzic A (2008b) Stem cells: biologics for regeneration. Clin Pharmacol Ther 84:620–623
Nelson TJ, Faustino RS, Chiriac A et al (2008c) $CXCR4^{+}$/$FLK\text{-}1^{+}$ biomarkers select a cardiopoietic lineage from embryonic stem cells. Stem Cells 26:1464–1473
Nelson TJ, Behfar A, Terzic A (2009a) Regenerative medicine and stem cell therapeutics. In: Waldman SA, Terzic A (eds) Pharmacology and therapeutics: principles to practice. Saunders/Elsevier, Philadelphia
Nelson TJ, Behfar A, Yamada S et al (2009b) Stem cell platforms for regenerative medicine. Clin Transl Sci 2:222–227
Nelson TJ, Martinez-Fernandez A, Terzic A (2009c) KCNJ11 knockout morula re-engineered by stem cell diploid aggregation. Philos Trans R Soc Lond B Biol Sci 364:269–276
Nelson TJ, Martinez-Fernandez A, Yamada S et al (2009d) Repair of acute myocardial infarction with human stemness factors induced pluirpotent stem cells. Circulation 120:408–416
Nelson TJ, Martinez-Fernandez A, Yamada S et al (2010) Induced pluripotent stem cells: advances to applications. Stem Cells Cloning 3:29–37
Nishikawa S, Goldstein RA, Nierras CR (2008) The promise of human induced pluripotent stem cells for research and therapy. Nat Rev Mol Cell Biol 9:725–729
Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448:313–317
Okita K, Nakagawa M, Hyenjong H et al (2008) Generation of mouse induced pluripotent stem cells without viral vectors. Science 322:949–953
Opie SR, Dib N (2006) Surgical and catheter delivery of autologous myoblasts in patients with congestive heart failure. Nat Clin Pract Cardiovasc Med 3(Suppl 1):S42–S55
Oyer PE, Stinson EB, Jamieson SA et al (1983) Cyclosporin A in cardiac allografting: a preliminary experience. Transplant Proc 15:1247
Papapetrou EP, Tomishima MJ, Chambers SM et al (2009) Stoichiometric and temporal requirements of Oct4, Sox2, Klf4, and c-Myc expression for efficient human iPSC induction and differentiation. Proc Natl Acad Sci USA 106:12759–12764
Park IH, Arora N, Huo H et al (2008a) Disease-specific induced pluripotent stem cells. Cell 134:877–886
Park IH, Lerou PH, Zhao R et al (2008b) Generation of human-induced pluripotent stem cells. Nat Protoc 3:1180–1186
Park IH, Zhao R, West JA et al (2008c) Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451:141–146
Perin EC, Dohmann HF, Borojevic R et al (2004) Improved exercise capacity and ischemia 6 and 12 months after transendocardial injection of autologous bone marrow mononuclear cells for ischemic cardiomyopathy. Circulation 110(Suppl 1):II213–II218
Quaini F, Urbanek K, Beltrami AP et al (2002) Chimerism of the transplanted heart. N Engl J Med 346:5–15
Raya A, Rodríguez-Pizà I, Guenechea G et al (2009) Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature 460:53–59
Rosamond W, Flegal K, Furie K et al (2008) American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics – 2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 117:e25–e146
Rose EA, Moskowitz AJ, Packer M et al (1999) The REMATCH trial: rationale, design, and end points. Randomized evaluation of mechanical assistance for the treatment of congestive heart failure. Ann Thorac Surg 67:723–730

Rosenthal N (2003) Prometheus's vulture and the stem-cell promise. N Engl J Med 349:267–274
Rupp S, Koyanagi M, Iwasaki M et al (2008) Characterization of long-term endogenous cardiac repair in children after heart transplantation. Eur Heart J 29:1867–1872
Sánchez PL, San Román JA, Villa A et al (2006) Contemplating the bright future of stem cell therapy for cardiovascular disease. Nat Clin Pract Cardiovasc Med 3(Suppl 1):S138–S151
Schächinger V, Assmus B, Britten MB et al (2004) Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI trial. J Am Coll Cardiol 44:1690–1699
Schächinger V, Erbs S, Elsässer A et al (2006) REPAIR-AMI investigators. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 355:1210–1221
Schenke-Layland K, Rhodes KE, Angelis E et al (2008) Reprogrammed mouse fibroblasts differentiate into cells of the cardiovascular and hematopoietic lineages. Stem Cells 26:1537–1546
Schneider JS, Vitale JM, Terzic A et al (2009) Blastocyst injection of embryonic stem cells: a simple approach to unveil mechanisms of corrections in mouse models of human disease. Stem Cell Rev Rep 5:369–377
Segers VF, Lee RT (2008) Stem-cell therapy for cardiac disease. Nature 451:937–942
Sha HY, Chen JQ, Chen J et al (2009) Fates of donor and recipient mitochondrial DNA during generation of interspecies SCNT-derived human ES-like cells. Cloning Stem Cells 11:497–507
Shi Y, Desponts C, Do JT et al (2008) Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. Cell Stem Cell 3:568–574
Silva J, Nichols J, Theunissen TW et al (2009) Nanog is the gateway to the pluripotent ground state. Cell 138:722–737
Srinivas G, Anversa P, Frishman WH (2009) Cytokines and myocardial regeneration: a novel treatment option for acute myocardial infarction. Cardiol Rev 17:1–9
Stadtfeld M, Nagaya M, Utikal J et al (2008) Induced pluripotent stem cells generated without viral integration. Science 322:945–949
Stillwell E, Vitale J, Zhao Q et al (2009) Blastocyst injection of wild type embryonic stem cells induces global corrections in mdx mice. PLoS One 4(3):e4759
Sun N, Panett NJ, Gupt DM et al (2009) Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. Proc Natl Acad Sci USA 106:15720–15725
Surani MA, McLaren A (2006) Stem cells: a new route to rejuvenation. Nature 443:284–285
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
Takahashi K, Okita K, Nakagawa M et al (2007a) Induction of pluripotent stem cells from fibroblast cultures. Nat Protoc 2:3081–3089
Takahashi K, Tanabe K, Ohnuki M et al (2007b) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
Takeuchi JK, Bruneau BG (2009) Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. Nature 459:708–711
Tam PP, Rossant J (2003) Mouse embryonic chimeras: tools for studying mammalian development. Development 130:6155–6163
Taylor DO, Edwards LB, Aurora P et al (2008) Registry of the International Society for Heart and Lung Transplantation: twenty-fifth official adult heart transplant report – 2008. J Heart Lung Transpl 27:943–956
Torella D, Ellison GM, Méndez-Ferrer S et al (2006) Resident human cardiac stem cells: role in cardiac cellular homeostasis and potential for myocardial regeneration. Nat Clin Pract Cardiovasc Med 3(Suppl 1):S8–S13
Urbanek K, Torella D, Sheikh F et al (2005) Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci USA 102:8692–8697
Utikal J, Polo JM, Stadtfeld M et al (2009) Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. Nature 460:1145–1148
Wakayama T, Tabar V, Rodriguez I et al (2001) Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science 292:740–743

Waldman SA, Terzic A (2007) Individualized medicine and the imperative of global health. Clin Pharmacol Ther 82:479–483
Waldman SA, Terzic A (2008) Therapeutic targeting: a crucible for individualized medicine. Clin Pharmacol Ther 83:651–654
Waldman SA, Terzic MR, Terzic A (2007) Molecular medicine hones therapeutic arts to science. Clin Pharmacol Ther 82:343–347
Wernig M, Zhao JP, Pruszak J et al (2008) Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. Proc Natl Acad Sci USA 105:5856–5861
Woltjen K, Michael IP, Mohseni P et al (2009) PiggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 458:766–770
Wood SA, Allen ND, Rossant J et al (1993a) Non-injection methods for the production of embryonic stem cell-embryo chimaeras. Nature 365:87–89
Wood SA, Pascoe WS, Schmidt C et al (1993b) Simple and efficient production of embryonic stem cell-embryo chimeras by coculture. Proc Natl Acad Sci USA 90:4582–4585
Xu D, Alipio Z, Fink LM et al (2009) Phenotypic correction of murine hemophilia a using an iPS cell-based therapy. Proc Natl Acad Sci USA 106:808–813
Yamada S, Nelson TJ, Behfar A et al (2009) Stem cell transplant into preimplantation embryo yields myocardial infarction-resistant adult phenotype. Stem Cells 27:1697–1705
Yamanaka S (2007) Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell Stem Cell 1:39–49
Yamanaka S (2008) Pluripotency and nuclear reprogramming. Philos Trans R Soc Lond B Biol Sci 363:2079–2087
Yamanaka S (2009a) A fresh look at iPS cells. Cell 137:13–17
Yamanaka S (2009b) Ekiden to iPS cells. Nat Med 15:1145–1148
Yamanaka S (2009c) Elite and stochastic models for induced pluripotent stem cell generation. Nature 460:49–52
Yang X, Smith SL, Tian XC et al (2007) Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nat Genet 39:295–302
Yang L, Soonpaa MH, Adler ED et al (2008) Human cardiovascular progenitor cells develop from a KDR^+ embryonic-stem-cell-derived population. Nature 453:524–852
Ye Z, Zhan H, Mali P et al (2009) Human induced pluripotent stem cells from blood cells of healthy donors and patients with acquired blood disorders. Blood 114(27):5473–5480
Yokoo N, Baba S, Kaichi S et al (2009) The effects of cardioactive drugs on cardiomyocytes derived from human induced pluripotent stem cells. Biochem Biophys Res Commun 387:482–488
Yu J, Vodyanik MA, Smuga-Otto K et al (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920
Yu J, Hu K, Smuga-Otto K et al (2009) Human induced pluripotent stem cells free of vector and transgene sequences. Science 324:797–801
Zhang J, Wilson GF, Soerens AG et al (2009) Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 104:e30–e41
Zhao XY, Li W, Lv Z et al (2009) iPS cells produce viable mice through tetraploid complementation. Nature 461:86–90
Zhou H, Wu S, Joo JY et al (2009) Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4:381–384

Chapter 21
Biodegradable Materials

Michael Schroeter, Britt Wildemann, and Andreas Lendlein

Abstract The ability of polymers to be degraded in physiological environments makes them interesting candidates for various medical applications. Degradation and metabolisation or excretion of polymeric implants can avoid a second surgery for the removal of an implant. Biodegradable materials can serve as a temporary substitute of the extracellular matrix or as matrix in controlled drug release systems, which both can be utilized in Regenerative Therapies.

This chapter gives an overview about polymeric materials established in clinical use such as polyesters, polyurethanes, polyanhydrides, or carbohydrates. It describes further their synthesis and exemplary applications such as surgical sutures. Finally the importance of a continuing development of novel materials for future applications is pointed out, since the number of potential applications in the medical field is expanding rapidly.

M. Schroeter, Ph.D.
Centre for Biomaterial Development, Institute of Polymer Research,
Helmholtz-Zentrum Geesthacht, Kantstr. 55, 14513 Teltow, Germany

B. Wildemann, Prof.
Julius Wolff Institute and Berlin-Brandenburg Centre for Regenerative Therapies (BCRT),
Charité – Universitätsmedizin Berlin, Campus Virchow-Klinikum, Augustenburger Platz 1,
Berlin 13353, Germany

A. Lendlein, Prof. (✉)
Centre for Biomaterial Development and Berlin-Brandenburg
Centre for Regenerative Therapies (BCRT), Institute of Polymer Research,
Helmholtz-Zentrum Geesthacht, Kantstr. 55, 14513 Teltow, Germany
e-mail: andreas.lendlein@hzg.de

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_21,

21.1 Introduction

Many people experience biomaterials in the form of dental fillings, contact lenses or suture materials. Further applications are artificial joints, blood vessel substitutes or drug delivery systems. A biomaterial is defined as any material intended to interact with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body (The European Society for Biomaterials 1991). Biomaterials can be inorganic materials like bioactive glasses, ceramics or metal alloys as well as polymers including natural polymers (e.g. collagen), synthetic polymers and combinations of both. Biomaterials can be applied in permanent or temporary implants, depending on the particular indication. While all biomaterials must be biocompatible their permanent application requires long term stability in physiological environments. An example for long term application is the acetabular cup in artificial hip joints. These materials need to be integrated into the surrounding tissue after implantation and retain their function for a long time. For temporary applications, biodegradable materials are demanded, being degraded and eliminated or metabolished by the organism in the course of time (Lendlein 1999). Biodegradation is defined as the gradual breakdown of a material mediated in or by a biological system. The advantage of the bodies' capability for self healing can be utilized by the use of degradable materials. A temporary implant is completely substituted by natural tissue in the best case. An overview about biodegradable polymers and natural materials, their synthesis and approved clinical applications will be given in this chapter.

Degradable biomaterials must include linkages, cleavable under physiological conditions. One possibility is the incorporation of hydrolytically degradable bonds (see Fig. 21.1).

Hydrolytic degradation has the advantage that water is generally available in the body. Therefore degradation should occur at different locations of application / implantation. In contrast, concentrations of enzymes can differ locally. As the degradation rate of hydrolytically cleavable bonds can be increased by enzymes substantially, degradation rate can differ significantly in different body parts or individuals. Chemical bonds whose cleavage is accelerated by enzymes can be used for the generation of local effects, such as the specific targeting of drugs or organ-specific processes. In general the advantage of devices made from degradable polymers is that a second surgery for explantation can be avoided.

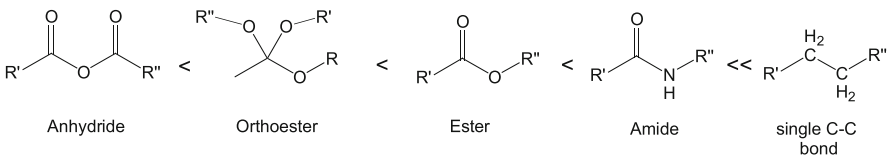


Fig. 21.1 Hydrolytically cleavable bonds in comparison to a carbon–carbon bond in order of their hydrolytic stability

Table 21.1 Parameters influencing the degradation rate of polymers

Structural parameters of macromolecules	Shaped body (device)	Environmental influence
Chemical composition	Processing conditions	location of implantation
Sequence structure in copolymers	Shape of the sample	Adsorbed or absorbed molecules
Presence of ionic groups	Sterilization	Ion exchange, -strength, pH-value
Branches/ chain defects	Thermomechanical "history" of the polymer	Changes of diffusion coefficient
Average molecular weight and distribution	Material inhomogenities and internal stress	Mechanism of hydrolytic degradation (H_2O, Enzymes)
	Surface roughness	Cracks due to hydrolytic degradation or mechanical tension

Two mechanisms for the hydrolytic biodegradation of polymers are discussed (i) bulk degradation and (ii) surface erosion. In case of bulk degradation the diffusion of water into a polymer matrix is faster than the hydrolysis rate. The hydrolytically cleavable bonds in the amorphous parts of the matrix can be degraded as water molecules are available because of fast diffusion (Brannon-Peppas 1997). Therefore, the average molecular weight of the matrix polymers decreases. In case of surface erosion the diffusion of water into the polymer is (much) slower than the degradation rate of the macromolecule. Hydrolysis is limited to a thin layer on the surface, while the molecular weight of the polymer in the bulk remains unchanged. In surface erosion the velocity of degradation depends on the shape of the sample. The higher the surface area is the higher is the rate of degradation. The number of hydrolytically cleavable bonds in a macromolecule affects the hydrolysis rate (see Fig. 21.1). Macromolecules containing orthoester or anhydride bonds as examples for easily hydrolysable bonds show a high tendency for surface erosion (Wu 1995). Many other parameters related to the polymer, the device (shaped body) or the environmental conditions can influence the degradation behavior of polymers (see Table 21.1).

The biodegradability of polymers can be determined *in vitro* and *in vivo*. For *in vitro* experiments the materials are exposed to an aqueous (buffer) solution, which may contain ions or to cell culture medium, which may contain amino acids, sugar as well as serum. Temperature and pH value can be varied to mimic specific situations and environments. The partially degraded materials as well as the degradation products can be isolated and characterized. The addition of specific enzymes is also possible (Kulkarni et al. 2007). *In vivo* experiments are performed with different species e.g. mice or rats to investigate the biodegradation and biocompatibility.

Standards exist for biocompatibility testing of materials used in the human body. The American Food and Drug Administration (FDA), the Health and Welfare Canada, and Health and Social Services UK introduced 1986 the "Tripartite Biocompatibility Guidance for Medical Devices". The guidance was developed to

help FDA reviewers, but also manufacturers of medical devices, in judging and selecting appropriate tests to evaluate the biological responses to medical devices. Four different device categories of biomaterials were defined: Non-Contact Devices, External Devices, Externally Communicating Devices, Internal Devices. The biological test of the materials include: Sensitization Assay, Irritation Tests, Cytotoxicity Tests, Acute Systemic Toxicity Tests, Hemocompatibility Tests, Hemolysis Tests, Implantation Tests, Mutagenicity (Genotoxicity)Tests, Chronic Toxicity Tests, Carcinogenesis Bioassay, Pharmacokinetics, Reproductive and Developmental Toxicity Tests. To harmonize the biocompatibility testing, the International Standards Organization (ISO) developed a standard for biological evaluation of medical devices (ISO 10993). Until today, this standard consists of 20 parts and the first part "Biological Evaluation of Medical Devices: Part 1: Evaluation and Testing" provides guidance for selecting the tests to evaluate the biological response to medical devices. The appropriate methods to conduct the biological tests are described in most of the other parts. The ISO 10993 is under permanent actualization and covers aspects of biomaterial testing.

21.2 Polymer-Based Biomaterials

Biodegradable polymers can be divided in two main groups: materials based on natural polymers, and purely synthetic polymers, designed to meet different demands.

Important groups of degradable polymers used in medical applications are: Polyesters, Polyesteramides, Poly(ortho ester)s, Polyurethanes, Polyanhydrides, Cyanoacrylates, Hydrogels (e.g. based on poly(ethylene glycol)).

Carbohydrates and proteins form the basis for many biomaterials based on natural polymers. Synthesis or isolation and exemplary applications of such materials are presented in the following.

21.2.1 Polyesters

An important group of biodegradable biomaterials are (co)-polyesters used in surgical sutures. The degradation of ester bonds occurs under hydrolysis of the bond, forming a carboxylic acid and an alcohol. The rate of hydrolysis depends on the neighboring groups to the ester. Polyester are typically prepared by ring-opening polymerization of lactones or cyclic diesters (Deasy et al. 1989; Piskin 1995; Vert 1986). A ring-opening polymerization proceeds in an anionic, cationic or coordination polymerization mechanism in the presence of catalysts and is started by initiators. Monomers like the cyclic diesters digylolide **1,** and dilactide **2**, as well as the lactones ε-caprolactone **3,** and β-butyrolactone **4** are frequently used. Further cyclic compounds, which can be polymerized in an analogue way, are cyclic carbonates (e.g. trimetylene carbonate, TMC **6**) dioxanone-compounds (e.g. *p*-dioxanone **7**),

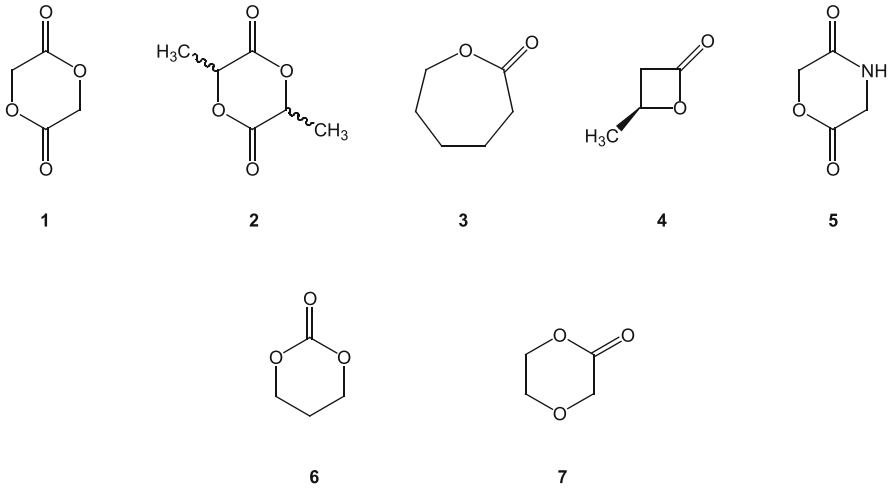


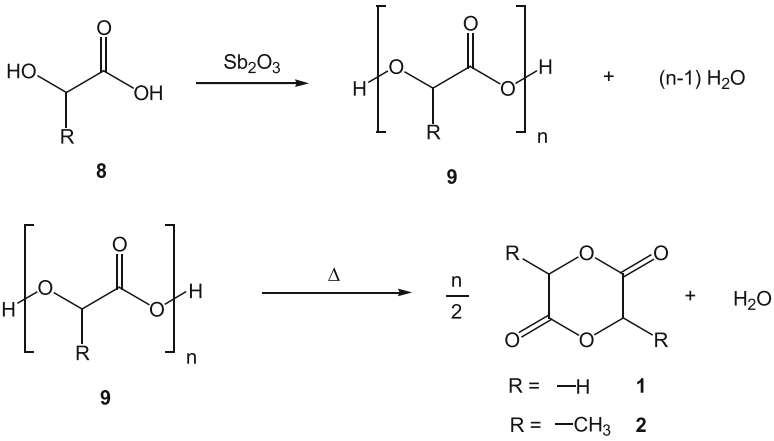
Fig. 21.2 Chemical structures of cyclic diesters and lactones used as (co)-monomers in the synthesis of degradable (co)-poly(ether)esters. **1**: diglycolide, **2**: dilactide, **3**: *ε*-caprolactone, **4**: *β*-butyrolactone, **5**: morpholino-2,5-dione, **6**: trimetylene carbonate, **7**: p-dioxanone

and compounds based on morpholino-2,5-dione **5** (see Fig. 21.2). As lactide provides two stereo centres three different isomers exist: *L,L*-dilactide, *D,D*-dilactide and *meso*-dilactide.

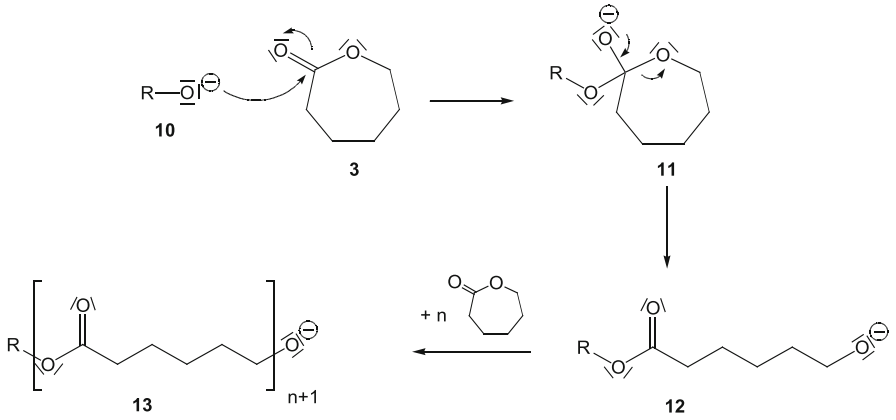
Cyclic diesters are generated from the corresponding hydroxyl carboxylic acids (Scheme 21.1). Oligoesters are formed by elimination of water in the presence of catalysts (e.g. Sb_2O_3). However, high molecular weight products can not be obtained by this process as the required high conversion rates were not reached.

The ring-opening polymerization of cyclic diesters can be performed as anionic polymerization or as coordination polymerization (Scheme 21.2). Sn (II) compounds like dibutyl-Sn-dilaurate are used as coordinative catalysts for the bulk polymerization (Leenslag and Pennings 1987). It has to be considered that such compounds catalyze transesterification reactions as well. Therefore, side reactions like inter- and intramolecular transesterification as well as depolymerization may occur. Heating of a mixture of two polyesters at 140°C will change the sequence structure of both polymers (Kricheldorf and Serra 1985; Nieuwenhuis 1992). As alternative catalysts Zn (II) ethylhexanoate (Leenslag et al. 1984), and Zn-powder (Chabot et al. 1983) have been studied. The application of these catalysts lead to polyesters of high molecular weight. Catalysts based on Magnesium and other metals are under development with the aim to decrease toxicity of the catalyst or facilitate its removal from the reaction mixture (Kricheldorf and Stricker 2000).

In addition to bulk polymerization polymerization processes in solution and in suspension are possible. The lower viscosity of the reaction mixture compared to a bulk process enables a better heat transfer and by that a better control of the reaction temperature.



Scheme 21.1 Synthesis of cyclic diesters by depolymerization of oligoesters at elevated temperatures



Scheme 21.2 Mechanism of anionic polymerization of ε-caprolactone

Homopolymers like poly(glycolic acid) (PGA), or poly(*L*-lactic acid) (PLLA), as well as copolymers of them with different comonomer ratios such as poly[(*rac*-lactid)-*co*-glycolide] (PLGA) were prepared by ring-opening polymerization. PGA and PLLA are semicrystalline polymers, whereas poly(*D,L*-lactic acid) (PDLLA) is amorphous. PLGA has a glass transition temperature (T_g) close to body temperature (T_g = 36°C), whereas PLLA and PDLLA have T_g values between 57 and 60°C and 50–54°C respectively (Vert 1989). PLLA has been studied as degradable biomaterial extensively (Tsuji et al. 2003; Ye et al. 2008).

Poly(ε-caprolactone) (PCL), prepared from ε-caprolactone **3**, is a semi-crystalline degradable polymer with sufficient mechanical strength and thermal stability for application as scaffold material or matrix material for drug delivery. The melting point of PCL is in the range of 59–64°C and its T_g is around −60°C.

The homopolymer is slowly degradable, due to high hydrophobicity and relatively low hydrolysis rate (Little et al. 2009).

Poly(*p*-dioxanone) (PPDO), synthesized from *p*-dioxanone **7** (Fig. 21.2), is a semi-crystalline degradable polymer with a melting point of 115°C and a T_g in the range between −10 and 0°C. Above its melting point this polymer depolymerizes to *p*-dioxanone **7** (Shalaby and Johnson 1994).

Several medical devices based on (co)-polyesters are applied in the clinic. The main products made out of polyester are sutures, orthopaedic implants and scaffolds (Nair and Laurencin 2007; Weigel et al. 2006). The FDA approved a suture called DEXON®, based on polyglycolide (Table 21.2) in 1969. Glycolide was further (co)-polymerized with TMC (PGATMC) and is on the market as a suture material (Maxon®).

(Co)-polyesters degrade mainly by bulk erosion. Due to water uptake random scission of polymer chains occurs in the amorphous domains. Oligomers and hydroxy acids are obtained as water soluble degradation products. The generated carboxy groups induce a autocatalytic process.

Despite the good results of the resorbable suture materials, concerns exist regarding the use of (co)-polyesters in ligament reconstruction surgery. A review article published in 2009 by Konan and Haddad summarized adverse reactions due to the use of resorbable screws in anterior cruciate ligament reconstruction surgery (Konan and Haddad 2009). They concluded that the resorbable materials offer advantages compared to metal screws, but also possible disadvantages, such as potential adverse biological responses resulting in the worst scenario, in a failure of the surgery. Further long term studies and the improvement of the material are necessary.

If polyesters are used as matrix material for drug delivery, the bulk erosion must also be considered (Li and Jastri 2006). The water uptake into the bulk material and the acidification might potentially interact with the drug. Several drug releasing implants have been developed (Table 21.2).

An injectable local drug delivery system was developed based on *in situ* forming implants. For this method a biodegradable, water insoluble polymer and the drug are solved in a non-toxic organic solvent. After injection the solvent dissipates into the tissue and water permeates into the polymer solution resulting in a precipitation and consequently the polymer forms an implant with the enclosed drug (Li and Jastri 2006). This principle was used for Atrigel and two products are already FDA approved: Eligard® (leuprolide acetate for injectable suspension) as a prostate cancer product that provide systemic release of leuprolide acetate for 1–4 month, and the Atridox® (8.5% doxycyline) for localized subgingival delivery of doxycycline.

Comparative clinical data about the performance of degradable materials versus materials not intended to degrade are rare. Dorri et al. performed a database search to analyze studies comparing the use of degradable versus titanium plates for facial surgery. They received 53 potentially eligible studies. However, none of the studies met the inclusion criteria. Based on these findings they concluded that the use of the plates should be based on the clinical experience and individual circumstances and those methodological sound trials are necessary (Dorri et al. 2009).

Table 21.2 Examples for devices -approved or in clinical trials- based on (co)-polyester

Implant material	Polymer	Name	Application
Sutures	PGA	Dexon®	All kinds of sutures (Gunatillake et al. 2006)
	PLGA	Vicryl® , Polysorb®	
	PDO	PDS®	
	PGATMC	Maxon®	
Screws	PLLA	Absolute®	ACL reconstruction (Purcell et al. 2004)
	ß-TCP/PLA	Biocryl®, Intrafix®	
	PLLA	Sheathed Femora®	
		BioScrew®	
		Bio-Cortical®	
		Bioabsorbable Wedge®	
	PLA	BioRCI®	
	PLLA/PGA	Gentle Threads®	
Plates & Screws	PLGA	RapidSorb®	Craniomaxillofacial surgery (Pietrzak and Eppley 2000; Peltoniemi et al. 2002)
	SR-PLDLA	Biosorb®	
	Copolymers of L-lactide, D,L-lactide, glycolide and TMC	Inion CPS® Fixation	
Skin and Cartilage	PLGA (Vicryl mesh)	Dermagraft®	Artificial skin
		NeoCyte®	Engineered cartilage (Ueda and Tabata 2003)
Anastomotic Ring	PGA and barium sulphate	Valtrac®	Anastomoses in the gastrointestinal tract surgery (Kaidar-Person et al. 2008)
Membranes	PLGA	Resolut®	Guided tissue regeneration (Greenstein and Caton 1993; Wolff and Mullally 2000)
		Vicryl Mesh®	
Facial surgery	PLLA	Sculptura®	Correction of facial fat loss
Nerve conduits	PGA	Neurotube®	
	PDLACL	Neurolac®	(Meek and Coert 2008)
Stents	PLLA	Igaki-Tamai® stent	Peripheral artery (Commandeur et al. 2006)

Drug delivery:			
Drug eluting stents	PLLA plus PDLLA coating and Everolimus	BVS everolimus-eluting stent	Coronary artery disease (Ormiston and Serruys 2009)
	Metal plus PLA coating and Biolimus A9™	BioMatrix®	Coronary artery disease or acute coronary syndromes (Commandeur et al. 2006)
Drug delivery	PLGA	LUPRON DEPOT® Zoladex	Release of gonadotropin releasing hormone agonist or goserelin acetate, benign gynaecological disorders; prostate/breast cancer
		Posurdex	Release of dexamethasone, retinal vein occlusion (Kuppermann et al. 2007)
	PLGA plus triclosan	Vicryl®plus	Antimicrobial sutures (Leaper et al. 2011)
	Titanium coated with PDLLA and Gentamicin	Expert Tibial Nail PROtect	Prevention of bacterial colonization on the implant (Schmidmaier et al. 2006)

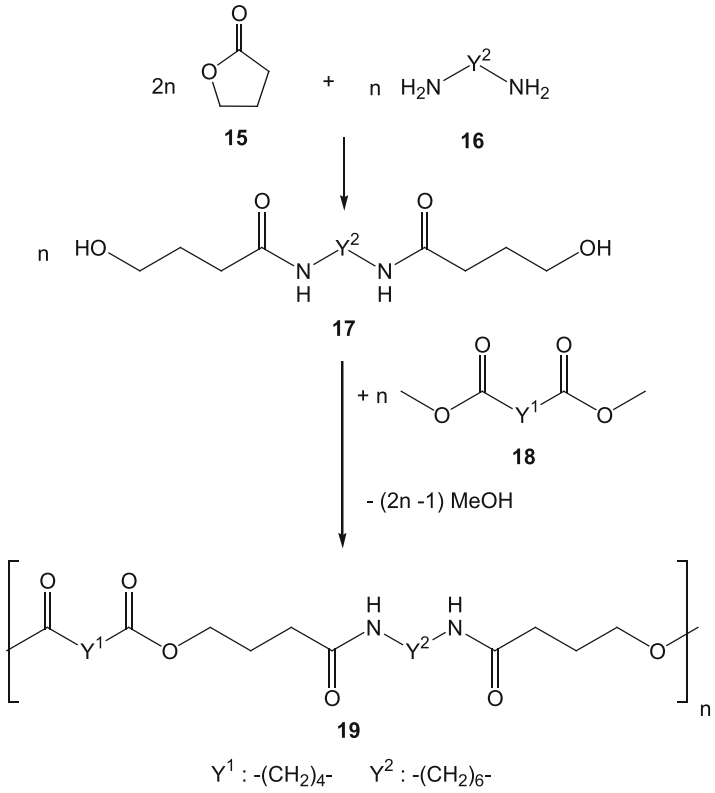
PGA poly(glycolic acid), *PLA* poly(lactide acid), *PLLA* poly(*L*-lactide acid), *PDLLA* poly(*D,L*-lactic acid), *PLGA* poly[(*rac*-lactid)-*co*-glycolide], *PCL* poly (ε-caprolactone), *PDLACL* Poly[(*rac*-lactide)-*co*-(ε-caprolactone)], *TMC* trimethylene carbonate, *PDO* polydioxanone, *ß-TCP* beta-tricalciumphosphate, *PGATMC* poly(glycolide-*co*-[trimethylene carbonate])

Very successful are degradable synthetic suture materials, which are in clinical use since 1969 with a huge market exceeding 1.3 billion dollar annually (Pillai and Sharma 2010). In general, degradable suture materials should be easy to handle, evoke only minimal tissue reaction, must not support bacterial growth, degrade after serving its function, and have an appropriate mechanical strength also during the degradation time period to support wound healing. A challenge in the development of suture materials is tailoring of the degradation time period and the change of the mechanical strength as well as suture elasticity during degradation, which needs to be adjusted to the clinical needs. Recently, developments of suture materials are focusing on self-knotting actions by usage of shape memory polymers, SMP (Lendlein and Langer 2002) and integration of bioactive compounds into the suture material, which have e.g. an antimicrobial activity (Vicryl®Plus, approved material) or inhibit matrix degradation (Pasternak et al. 2008).

21.2.2 Poly(ester amide)s

Poly(ester amides) (PEAs) can be prepared by different synthetic routes, which yield polymers with segmented, statistical distribution of chain segments. A random copolymer can be derived from 1,4-butanediol, adipic acid and ε-caprolactame (Grigat et al. 1998). The mechanical properties of segmented PEAs (and also of polyurethanes) are interesting because of the microphase separation of their hard and soft segments. In PEA soft domains were formed by the ester-rich domains, and hard domains are formed by the amide-rich domains acting as physical crosslinkers determining the shape of a sample body. A segmented PEA could be synthesized by reaction of an alternating ester-amide oligomer, obtained from the reaction of adipic acid with a bisamide diol derived from 1,6-diaminohexane (**16** with Y^2=C6) and γ-butyrolactone **15**, with an oligoester prepared from 1,2-ethanediol and dimethyl adipate (Bera and Jedlinski 1993, Scheme 21.3).

There are four types of biodegradable PEAs: (a) Polydepsipeptides, which combine properties of poly(α-hydroxy acids) and poly(α-amino acids). These polymers can be prepared by ring-opening polymerization of morpholine-2,5-diones (see **5** in Fig. 21.2) (Feng and Guo 2009). (b) Derivatives of α-hydroxy acids obtained by reaction of an acid dichloride with a bisamide diol prepared from glycolic acid and diaminoalkanes. The polymers showed promising results in mechanical properties, degradability, and biocompatibility (Horton et al. 1988); (c) Derivatives from α-amino acids: Poly(ester amides) containing α-amino acid units have been developed and extensively studied (Guo and Chu 2007). These polymers can be obtained by polymerization of an acid dichloride and the p-toluenesulfonic salt of a bis (α-amino acid) α,ω-alkylene diester (Paredes et al. 1998). This polymer type has the disadvantage of relatively high production costs, insolubility in common organic solvents, and thermal instability (Vera et al. 2006); (d) Polymers made from carbohydrate derivatives: carbohydrates like arabinose, xylose and tartaric acid have been used for the formation of polymers by reaction with amins and esters and their degradation



Scheme 21.3 Synthesis of a PEA

properties were investigated (Martinez et al. 1997). The degradation is strongly influenced by the chain microstructure of the resulting polymer. The ester moieties degrade faster than the amide moieties, so that the degradation rate can be adjusted by the amount of ester moieties in the copolymer.

21.2.3 Poly(ortho ester)s

Poly(ortho ester)s (POEs) were developed for drug delivery applications. Four types of POEs have been developed, which are shown in Fig. 21.3.

POE II is prepared by reaction of diketene acetal **20** (DETOSU) with an appropriate diol **21** (see Scheme 21.4) (Heller et al. 1992). DETOSU was synthesized from the corresponding diallyl pentaerytritol (Crivello et al. 1996).

This type of poly(ortho ester) was investigated as a drug delivery system for Ivermectin containing strands to prevent heartworm infestation in dogs using a cross-linked matrix containing a trivalent alcohol as cross-linker (Shih et al. 1993). The degradation behavior was not sufficiently predictable.

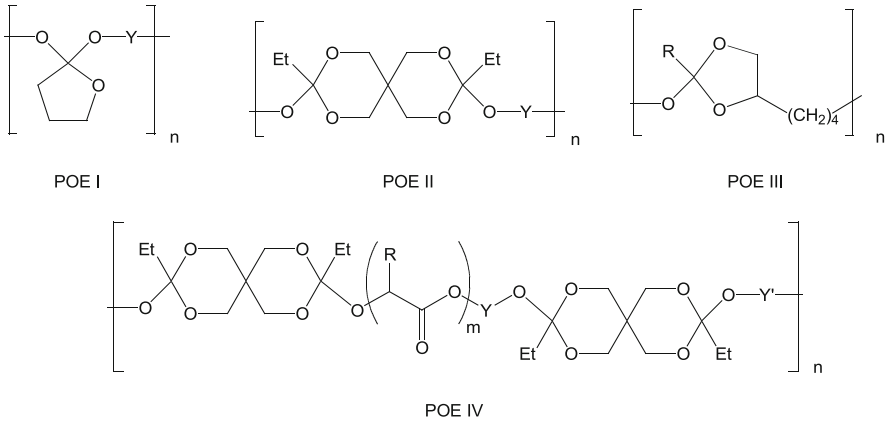
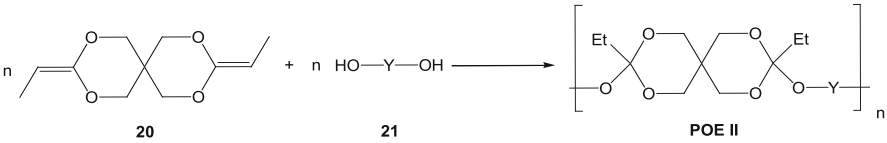


Fig. 21.3 Chemical structures of the four types of poly(ortho ester)s



Scheme 21.4 Synthesis of POE Type II

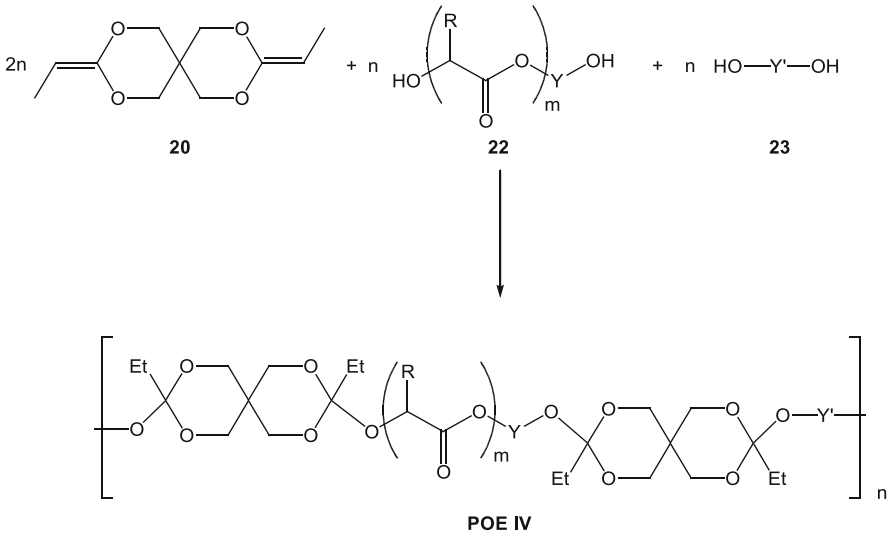
POE IV is prepared by the reaction of DETOSU, a mixture of diols and an acid diol as shown in Scheme 21.5 (Ng et al. 1997). Here, concentration of the α-hydroxy acid segments in the polymer chains controls the degradation rate (Y = alkyl).

The mechanical properties of the polymers (POE II and IV) can be influenced by using rigid diols such as *trans*-cyclohexandimethanol and flexible diols like 1,6 hexanediol (Y′ = C6). The T_g is determined by the ratio of such diols in the polymer (Heller et al. 1983, 1995). For drug delivery systems the drug has to be uniformly distributed over the polymer matrix. POE IV can be processed by melt extrusion at 100°C without significant change in molecular weight. POE II and IV are soluble in solvents like methylene chloride, ethyl acetate, or THF, enabling formation of microspheres by conventional procedures.

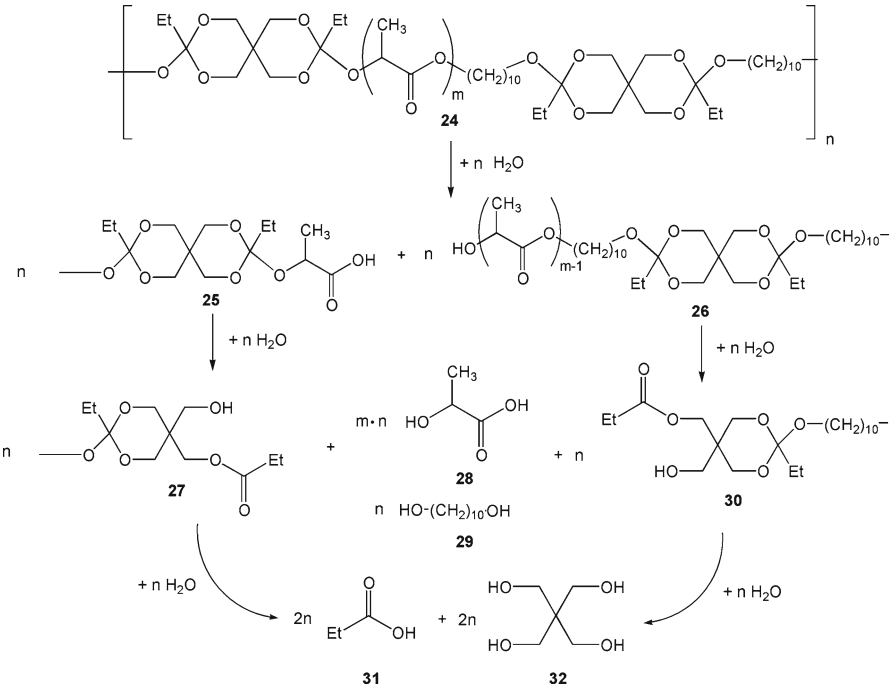
Poly(ortho ester)s are stable when stored under water-free conditions at room temperature and can be sterilized (Heller et al. 2002).

The hydrolysis of the POE IV proceeds in three consecutive steps (see Scheme 21.6).

The weight loss during degradation is linear for POE Type IV. First the ester bonds were cleaved in the polylactide moiety of the polymer (leads to **25**, **26**) and as a second step the orthoester moiety degraded. This resulted finally in the release of lactic acid **28**, propionic acid **31**, pentaerytritol **32** and decandiol **29**. The process is predominantly confined to the surface layers of the polymer matrix (surface erosion). Only a small amount of bulk erosion occurs, which is in contrast to the poly(lactide-*co*-glycolide) copolymers or poly(lactic acids) (Vaccaro et al. 2002).



Scheme 21.5 Synthesis of POE Type IV



Scheme 21.6 Degradation mechanism of POE type IV in the presence of water

Hydrolytically labile poly(ortho ester amide) (POEA) copolymers were developed to overcome the drawbacks of the traditional methods of POE synthesis by solution polycondensation between an acid labile diamine with a build-in ortho ester bond and fatty diacid esters of different chain-length (Tang et al. 2009).

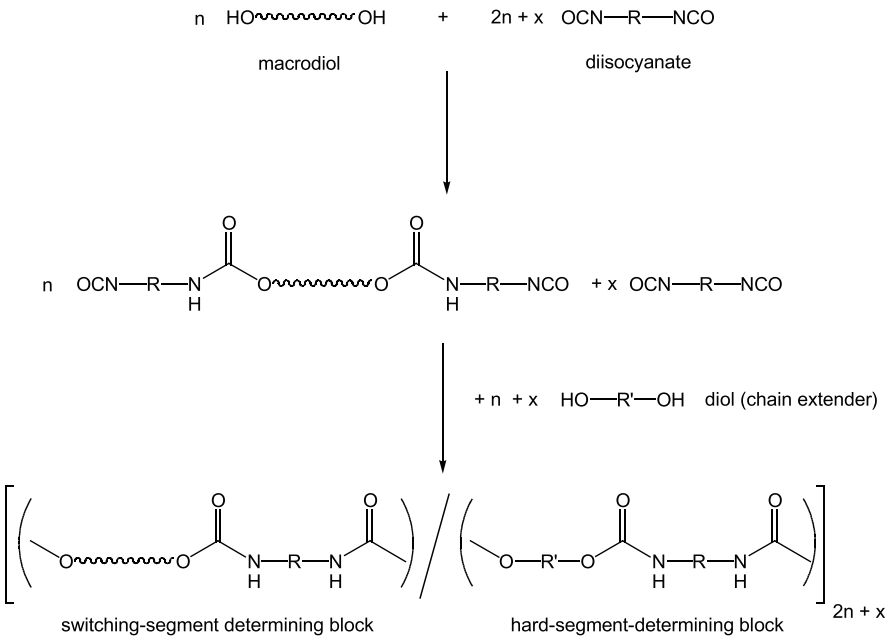
21.2.4 Polyurethanes

Polyurethanes (PURs) are used for industrial applications since the 1940s, but development of biocompatible polymers based on urethanes started in the 1960s. These polymers are often used for long-term applications because of their beneficial characteristics like toughness, durability and biostability. Also polyurethanes with a controlled degradation rate have been developed due to a high demand on degradable biomaterials (Lendlein et al. 1998).

PURs are basically synthesized using a diisocyanate, a diol and a chain-extender as main components (Syzcher 1999). In these cases aromatic diisocyanates were substituted by an aliphatic compound such as 1,6-hexamethylene diisocyanate (HDI), 1,4-butylene diisocyanate (BDI), lysine methylester diisocyanate (LDI), or trimethyl hexamethylene diisocyanate (TMDI) (Cardy 1979, Scheme 21.7).

The diol in degradable PURs is commonly an oligomer with hydroxyl end groups, so called macrodiol, with a backbone corresponding to polyester or polycarbonate. Polyester urethanes are the most common degradable polymers of this type. The macrodiols can be prepared by ring-opening polymerization of a cyclic lactone (see Sect. 21.2.1). The reaction between the diol and the isocyanate is carried out with an excess of diisocyanate to obtain a reactive prepolymer with isocyanate end groups. To obtain a thermoplastic PUR with a segmented architecture the prepolymer is further reacted with a chain extender, which is a short chain diol.

PURs are multi-block copolymers, which show microphase separation. This phase separation comparable to PEAs allows another functionality beside degradability in the materials: these polymers show shape-memory properties. Using poly(*ε*-caprolactone)diol and poly(*p*-dioxanone) together with TMDI a degradable SMP can be generated (Lendlein and Kelch 2002; Lendlein and Langer 2002; Spaans et al. 1998). SMP are materials, which can be deformed and fixed in a temporary shape, from which they recover their original shape only when exposed to an appropriate stimulus (Behl and Lendlein 2007). They show at least two separated phases. The phase with the highest thermal transition acts as a physical cross-link and is responsible for the so called permanent shape of the polymer. A second phase serves as a molecular switch and enables the fixation of a temporary shape. The transition temperature T_{trans} for the fixation of the switching segments can either be a T_g or a T_m. After forming the material above the switching temperature, the temporary shape can be fixed by cooling the polymer below the switching temperature. Heating the material above T_{trans} cleaves again the physical crosslinks in the switching phase. As a result of its entropy elasticity the material is forced back to its permanent shape. Potential applications are intelligent degradable sutures and degradable



Scheme 21.7 Synthesis of a polyurethane using a macrodiol, a diisocyanate and a short chain diol as a chain extender

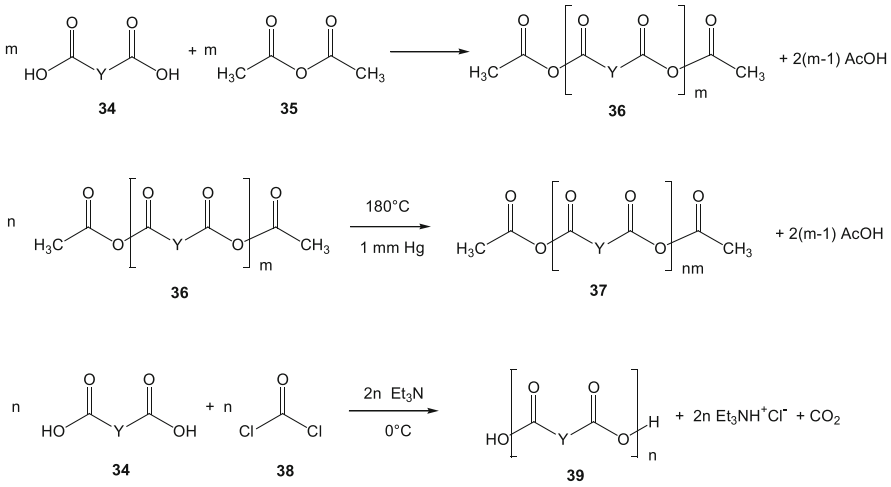
shape-memory-stents. Degradation is controlled by the amount of degradable bonds (e.g. ester bonds) in the used macrodiols for synthesis.

Degradable PURs are of interest in the design of scaffolds for in vivo tissue engineering as well as for cardiovascular applications. Elastic materials are required for soft tissue engineering due to mechanical conditions during the development of the new tissue. For cardiovascular tissue engineering the material should have sufficient elasticity and high tensile strength.

21.2.5 Polyanhydrides

A group of polymers showing surface erosion are the polyanhydrides (Bucher and Slade 1909; Hill and Carothers 1932; Domb et al. 1994; Laurencin et al. 1995). Since beginning of the 1980s polyanhydrides are developed for biomedical applications. The easily cleavable anhydride bond is introduced into a hydrophobic polymer, such as aliphatic long chain diacids (such as **34**). For variation of the mechanical properties by adjusting the crystallinity, sebacinic acid is often used as a comonomer.

Aliphatic diacids can be polycondensated to polyanhydrides by reaction with acetic acid anhydride **35** (Scheme 21.8). The reaction proceeds in two steps. First



Scheme 21.8 Synthetic routes for the synthesis of polyanhydrides

oligomeric polyanhydrides with terminal acetate groups are received (**36**), further reaction to high molecular product occurs at elevated temperatures under vacuum. When glutaric acid (Y = C10) and succinic acid instead of sebacic acid are used under the same conditions, they form cyclic compounds. The reaction between dicarboxylic acids and dicarboxylic diacidchlorides results in low molecular weight products. To gain higher molecular weight products phosgene (**38**) is used as condensation agent. Et_3N is used as a proton acceptor and precipitates the evolving hydrochloride.

While polysebacinic acid is semi-crystalline (T_m = 82°C) the homopolymer of the oleic acid dimer **40** is liquid. Copolymers of them are partly crystalline with T_m between 30 and 78°C having average molecular weights between 24.000 and 280.000 g mol^{-1}. Sebacinic acid **41** can be condensed with benzoic acid derivative **42** to form the drug delivery matrix Septacin® for curing chronic bone infections (Fig. 21.4, see applications).

It is assumed that polyanhydrides degrade by surface erosion mainly driven by two processes: (a) the easily hydrolysable anhydride bonds at the surface, and (b) the restriction of water permeability into the bulk due to hydrophobicity (Jain et al. 2005). These two processes allow a control of the release and a protection of the drug within the bulk material until release. In addition, the release of the drug is timely correlated to the material degradation. The duration of the polymer degradation can be controlled by varying the type of monomer and the comonomer ratio. Various polyanhydrides have been used experimentally as drug delivery systems (Table 21.3). As a localized drug delivery system for chemotherapeutic agents GLIADEL® is used in brain cancer treatment. The first approval in 1996 was for its limited use as an additive therapy in patient with

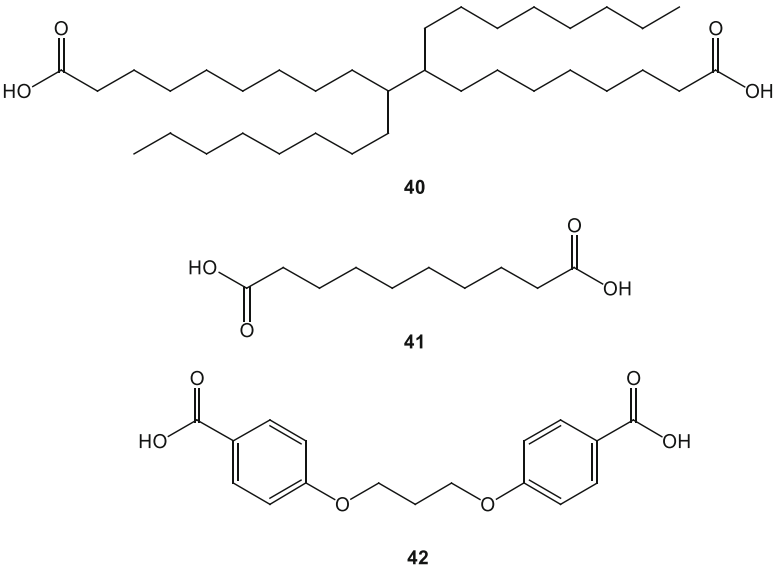
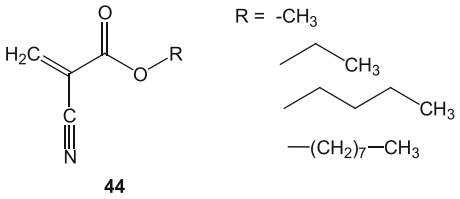


Fig. 21.4 Different diacids used as monomers in the preparation of polyanhydrides

Table 21.3 Experimentally used polyanhydrides for drug delivery (taken from Jain et al. (2005))

Delivery System	Polyanhydride	Drug	Disease
Matrix	Ricinoleic acid based	Methotrexate	Cancer
	P(RA- SA)	Cisplatinum	Cancer
	P(FAD-SA)	Cisplatin, 5-FU, methotrexate, paclitaxel	Cancer
	P(FAD-SA)	Bupivacaine HCL	Local anesthesia
	P(OA/LAD-SA)	Gentamicin	Osteomyelits
	P(DDDA-TA)	Ciprofloxacine hydrochloride	Local infection
Implant	P(CPP-SA)	BrdU & N-(phosphonacetyl)-l-aspartic acid; 5-fluorouracil or Camptothecin	Cancer
	P(CPP-SA)	Dibucaine, bupivacaine	Local anaesthesia
	P(CPP-SA)	Etoposide	Glaucoma
	P(FAD-SA)	Taxol	Cancer
	P(EAD-SA)	Heparin	Restenosis
Injectable paste	P(RA-SA)	Paclitaxel	Cancer
Microspheres	Poly(anhydride-esters)	Aminosalicylates	Inflammatory bowel disease
	PLA-PSA-PLA	Triamcinolone	Inflammation
	P(FAD-SA)	GnRHa	Hormone therapy
	SA copolymers	Bethanechol	Alzheimer disease

P(BA-PA) Poly(brassylic acidpentadecandioic acid), *P(CPP-SA)* Poly[1,3-bis(*p*-carboxyphenoxy) propane-*co*-sebacic anhydride], *P(DDDA-TA)* Poly(dodecane dioic acid-*co*-tetradecanedioic acid), *P(EAD-SA)* Poly(erucic acid-*co*-dimersebacic acid), *P(FAD-SA)* Poly(fatty acid dimer-*co*-sebacic acid), *PLA-PSA-PLA* Poly(lactic acid)-poly(sebacic acid)-poly(lactic acid), *P(OA/LAD-SA)* Poly(oleic acid/linoleic acid dimer-*co*-sebacic acid), *P(RA-SA)* Poly(ricinoleic acid-*co*-sebacic acid), *SA* Sebacic acid



44

Fig. 21.5 Chemical structure of cyanoacrylates

recurrent Glioblastoma multiforme (GBM) for whom surgical resection is indicated. In 2003, the approval was expanded for use of Gliadel® in patients with newly diagnosed high-grade malignant gliomas, as an adjunct to surgery and radiation. SEPTACIN® is a Gentamicin delivering product for osteomyelitis treatment (Li et al. 2002).

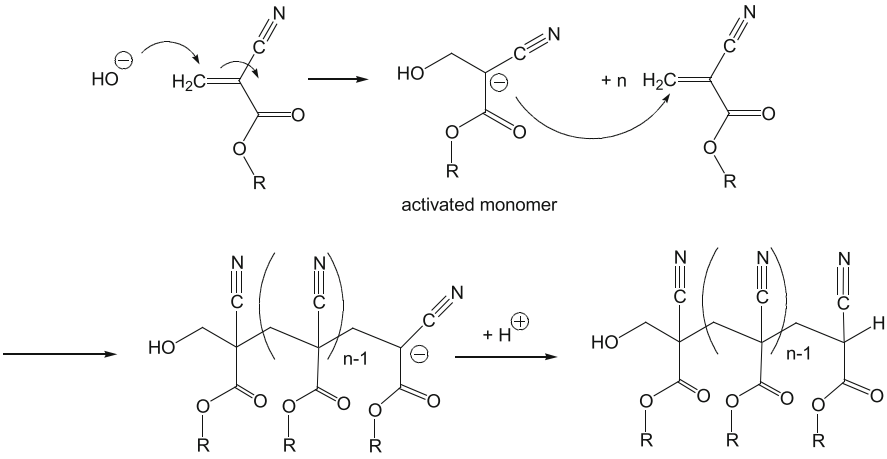
21.2.6 Polycyanoacrylates

Cyanoacrylate is a generic name for fast acting glues based on various cyanoacrylates such as methyl-2-cyanoacrylate, ethyl-2-cyanoacrylate, n-butyl-2-cyanoacrylate, and octyl-2-cyanoacrylate (see **44**, Fig. 21.5). These polymers are suitable for bonding tissue, and have been exploited for the benefit of suture-less surgery (Scheme 21.9).

Cyanoacrylates rapidly polymerize in the presence of traces of water (specifically hydroxyl ions), forming polymers with chain length sufficient for the demanded physical properties. Such polymers are able to join surfaces of different roughness.

N-butyl, isobutyl, and octyl ester derivatives of the cyanoacrylates were used in medical and veterinary applications. They are considered bacteriostatic. Polymers made from *n*-butyl monomers are rigid; octyl ester containing polymers provide more flexible materials. The polymer generated from octyl-2-cyanoacrylate degrades more slowly compared to formulations from shorter alkyl ester chains. Degradation products remain below the threshold of tissue toxicity, if the polymers degrade slowly. The degradation of the cyanoacrylates happens via an unzipping mechanism of the polymer, which proceeds by a retro-Knoevenagel reaction after elimination of the hydroxyl group. The ester bonds in the structure can be cleaved by acetic or basic pH (Han et al. 2008).

Cyanoacrylates based products are: Dermabond®, LiquiBand®, SurgiSeal™, or Nexaband® (all 2-octyl cyanoacrylate) and Indermil® and Histoacryl® (both are *n*-butyl-cyanoacrylates). All products are approved for topical use only. They serve as adjuncts to closure of skin incisions and Dermabond® and Indermil® are also applied as barrier to bacterial skin penetration (Spotnitz and Burks 2008).



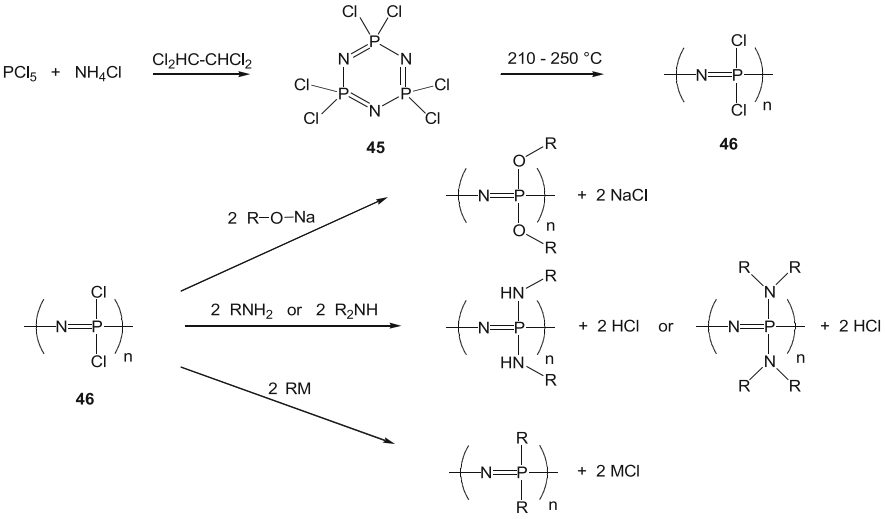
Scheme 21.9 Polymerization mechanism of cyanoacrylates in the presence of water

21.2.7 Polyphosphazenes

Polyphosphazenes are a class of biocompatible polymers (Andrianov 2009), which are prepared by reaction of phosphorus pentachloride with ammonium chloride in tetrachloroethane forming Hexachlorocyclotriphosphazene **45** in a first step. After heating to 210–250°C the chlorine substituted polymer **46** forms by thermal ring-opening polymerization (Allen 1981). In a second step the polymer is functionalized by nucleophilic attack on the phosphor (see Scheme 21.10) in solutions with benzene, toluene or tetrahydrofurane. A high variety of functional groups can be introduced such as amines, amino acids, poly(ethylene glycol)s or aliphatic and aromatic chains. The hydrophilic substituted polymers are able to degrade to phosphate, ammonia and an organic residue depending on the functionalization of the backbone. Phosphate and ammonia create a pH buffer system during degradation. Aromatic or aliphatic substitution leads to durable polymers.

The properties of the polymers depend on the nature of the side groups. With side groups derived from trifluoroethanol ($-O\text{-}CH_2\text{-}CF_3$) the polymers show high flexibility and a low T_g. In this respect the polymers resemble the commercial significant siloxanes.

Polyphosphazenes are explored as degradable scaffolds for bone regeneration in tissue engineering (Lakshmi et al. 2003). Here the polymers are functionalized with amino acid ethyl esters and can be electrospun to generate a non-woven scaffold. The degradation products are aside of phosphate and ammonia the amino acid ethyl esters. Introduction of carboxylic groups enable ionic crosslinking with calcium ions. Also an interconnected 3D porous scaffold for TE applications was generated by blending polyphosphazene with a hydrophilic glycylglycine dipeptide and a



Scheme 21.10 Synthesis and chemical functionalization of polyphosphazenes

hydrophobic 4-phenylphenoxy substituent in the backbone together with PLGA. The polyphosphazene self-assembles to microspheres of 10–100 μm (Deng et al. 2010). Furthermore further bioerodable blends of the polyphosphazenes with e.g. PLGA have been developed (Krogman et al. 2006).

The same type of polyphosphazene can be used as a drug delivery device in the form of nano- or microparticles (Sethuraman et al. 2011). They show a long blood circulating life time and are PEG coated.

21.2.8 Hydrogels

Hydrogels are three dimensional networks from hydrophilic polymer chains, which are able to take up high amounts of water under retention of their shape. The networks can be based on physical or covalent cross-links. Potential applications include matrices for cell culturing or drug delivery systems (Hoffman 2002; Peppas 1987). Hydrogels suitable for long term application are approved as soft contact lenses, made from 2-hydroxyethylmethacrylate and a cross-linker of PEG with reactive end groups. Hydrogels can be designed to be stimuli-sensitive by introduction of specific functional groups or segments (Qiu and Park 2001).

Degradability can be established by introduction of degradable blocks like PLA in the main chain of the hydrogel.

Examples for approved products are CoSeal™ (for vascular sealing) and DuraSeal™, (for dural sealing). Both of these sealants are synthetic and form hydrogels that seal tissues. CoSeal™ is indicated as an adjunct to blood vessel hemostasis by mechanically

sealing areas of leakage. DuraSeal™ consists of two solutions. The first is a PEG ester and the second contains trilisine amine with a blue dye for visualization. Following normal dura suturing the use of DuraSeal™ allows a true watertight closure.

21.3 Biomaterials Based on Natural Products

Carbohydrates are isolated from different natural sources. Hyaluronic acid, a non-sulfated glycosaminoglycan and one of the main components of the extracellular matrix, was isolated e.g. from cock's combs (Boas 1949), or isolated by gel filtration on agarose together with the protein complex (Barker and Young 1966) and is nowadays isolated from various sources. The repeating unit is a disaccharide composed of D-glucuronic acid and D-*N*-acetylglucosamine, linked together via alternating β-1,4 and β-1,3 glycosidic bonds (4GlcUAβ1-3GlcNAcβ1) (see Fig. 21.6). Hyaluronic acid is degraded enzymatically by hyaluronidases. In humans, there are at least seven types of hyaluronidase-like enzymes.

In the human body the polysaccharide hyaluronic acid (HA) is found in almost every tissue and half of the total HA content in the body is present in the skin. The main clinical application is the use of esterified HA as a wound dressing (HYAFF®) (Nair and Laurencin 2007). In orthopaedic surgery, hyaluronic acid scaffolds are used experimentally and in clinical trials as carrier for stimulating factors and cells (HYAFF®11, HYALOGRAFT C®). Viscous HA is used as a synovial substitute in osteoarthritis patient for pain relief and to improve joint mobility (SYNVISC® ORTHOVISC®).

Chitin and Chitosan are carbohydrates, which are FDA approved as food additives. Two other products are approved as medical device: CHITOSKIN® is a wound dressing and CHITOSTYPE® is used to reduce bleeding.

Polysaccharide spheres are the basis of the absorbable hemostat Vitasure®. Natural hemostasis is enhanced by the spheres that act as hydrophilic molecular sieves concentrating blood solids (platelets, red blood cells, and blood proteins) on the particle surfaces to form a gelled matrix. This gel matrix reduces further blood loss and is formed regardless of the patient's coagulation status.

Alginic acid is a polysaccharide of brown algae. As an alternative bone grafting material, ALGISORB™ is available. After cleaning and manufacturing the algae is transformed into calcium phosphate a major inorganic component of bone.

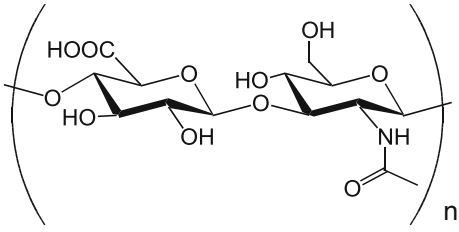


Fig. 21.6 Chemical structure of hyaluronic acid

21.3.1 Other Natural Materials

Natural materials have been used for centuries and have a broad range of applications in human medicine. In addition to autologous (patient's own) material, allogenic (human donor) and xenogenic (animal donor) transplant material, including various collagen products are currently used (Table 21.4). Furthermore non-animal materials, for example from algae, are also approved for human application (see carbohydrates).

21.3.2 Examples for Natural Materials

Collagen is the main protein of connective tissue and the most abundant protein in mammals (Di Lullo et al. 2002). Twenty nine types of collagen have thus far been identified and described in literature. Over 90% if the collagens in the body are of so called type I, II, III, and IV. Different methods are used to isolate collagen from tissues [for example see (Xiong et al. 2007)].

A large number of collagen products are on the marked. Collagen is clinically used, for example as a nerve conduit NeuraGen® and NeuroMatrix™ (bovine) and received FDA approval in 2001 (Meek and Coert 2008).

In combinations with osteoinductive growth factors (BMP-2 or BMP-7), bovine collagen is used as a carrier in the form of a sponge (InductOs®) or as granules with a particle size of 75–425 μm (Osigraft®) (Friedlaender et al. 2001; Govender et al. 2002).

KOLLAGEN-resorb®, GENTA-COLL resorb®, GentaFleece® and Septocoll® for example have several indications in surgery and are used for hemostasis, as a wound dressing, defect filler, and for bone regeneration. The supplementation of the collagen by adding gentamicin allows for protection against infections.

A product group based on natural porcine small intestinal submucosa (SIS) is marketed under the name Surgisis (SIS®) (Hodde 2006) for the treatment in: congenial diaphragmatic hernias (CDH), colon and rectal surgery, gastroenterology, general surgery, obstetrics & gynaecology, otolaryngology, plastic surgery, thoracic surgery, urology, vascular surgery. Surgisis is an acellularized matrix mesh composed of collagen, proteins, glycosaminoglycans, and proteoglycans.

A gelatinous protein mixture secreted by Engelbreth-Holm-Swarm sarcomosa cells (EHS) is commercialized under the name BD Matrigel™. This mixture resembles the complex extra cellular environment found in many tissues and contains laminin, entactin, and collagen. These proteins self assemble to a structure, which enables coating of glassware and 3D scaffolds for tissue engineering (Hughes et al. 2010).

Open porous collagen scaffolds under the name Optimax® are available for a drop in or drop on cell seeding. They are stable in cell culture for several weeks and

Table 21.4 Overview on tissue grafts

	Source/Organ	Application	
Autograft	Bone	Defect filling	For detailed information see Part II
	Mesenchymal stem cells	Various applications	
	Tendon	Tendon repair	
	Skin	Wound repair	
	Cartilage	Cartilage repair	
	Vessels aorta; coronary artery	Vessel replacement	
Allograft	Bone: spinal fusion grafts, cortical and dense cancellous bone, demineralized bone matrix	Defect filling, Spinal fusion, Periodontal Surgery	From tissue banks, processed materials
	Tendon	Tendon repair	
	Split Thickness Skin	Wound repair	
	Acellular dermis	Hernia repair and abdominal wall reconstruction.	
	Liver	Liver transplantation (TX)	Live donations, unprocessed
	Kidney	TX	
	Heart	TX	
	Lung	TX	
	Pancreas	TX	
	Skin, cornea	TX	
	Bone Marrow, stem cells	Leukemia	
Xenograft	Cardiac valve	Heart surgery	Mainly porcine, processed
	Collagen	Various applications	Bovine, porcine, equine, processed
	Bone	Defect filling	Mainly bovine, processed
Mixed	Fibrin human fibrinogen and human thrombin	Tissue sealant	
	Gelatine	Hemostatic	

are used for drug production in bioreactors, cell expansion, and tissue engineering application in preclinical development. The oriented pore structure in the scaffold enables directed growth of e.g. muscle cells in cell cultures (Kroehne et al. 2008). The pores are generated from controlled freezing of a water suspension of collagen, where the collagen molecules orient on the surface of finger like ice crystals. In the final step the ice is removed by freeze drying. Furthermore a resorbable dental barrier membrane (Remaix®) for applications in guided bone regeneration (GBR) and guided tissue regeneration (GTR) prepared from highly purified porcine collagen fibers intermingled with highly purified elastin fibers is available. The membrane is used to cover the space filled with bone graft material and assists the regeneration by protecting the slowly growing bone from infiltration with cells from the surrounding soft tissue.

Several materials of natural origin are available as hemostats to reduce or stop bleeding due to surgery. Sponges or meshes made from porcine gelatine (Gelfoam sponge®, Surgifoam sponge/powder®) or bovine collagen (Avitine sponge/flour®, Helistat® & Helitene®, Instat®) function as a mechanical barrier. To actively stop bleeding, active substances based on thrombin are approved. These materials can be of bovine origin (Thrombin JMI®), made from human thrombin (Evithorm®) or be recombinant (Recothrom®) (Spotnitz and Burks 2008).

Fibrin is also used as a hemostat and sealant (Tisseel®, Evicel®). It is a combination of plasma fibrinogen and thrombin from human or bovine origin. A dual chamber syringe separates the thrombin and the fibrinogen and after mixture of thrombin with the fibrinogen, a fibrin clot forms. Cryoseal® Fibrin Sealant System is a semi-automated product designed to produce an autologous fibrin sealant during surgery. Vitagel® is a combination product of microfibrillar collagen and thrombin in combination with the autologous plasma (fibrinogen and platelets).

21.4 Conclusion and Outlook

For each application of degradable polymers a specific set of properties e.g. mechanical properties such as degradability or a certain modulus is demanded. With increasing number of potential applications a greater extend of various materials with different property combinations is required. It is still only a limited amount of materials in clinical application, which are not able to fulfil all the new requirements. Therefore a substantial need of novel degradable biomaterials with tailored properties exists. Additional to the purely synthetic materials biomimetic approaches are integrated into material design.

Emerging fields of modern medicine, e.g. regenerative medicine require materials with a variety of several functionalities (e.g. shape-memory effect or other stimuli sensitive functions) combined in one material. Hence multifunctional materials are an important research topic. One example is a degradable shape-memory polymer with the additional ability of controlled drug release.

Multifunctional materials will be designed for the microenvironment of cells, e.g. mesenchymal stem cells. The fate of stem cells could be potentially controlled by generation of a functional mimic of this environment, which would be of high significance for Regenerative Therapies.

For a successful development of new materials a solid knowledge of existing applied polymers in clinical use is necessary to avoid old pitfalls and enable new combinations.

Acknowledgements We would like to thank the Deutsche Forschungsgemeinschaft (DFG, SFB 760) and the Bundesministerium für Bildung und Forschung (BMBF) for supporting the interdisciplinary research in the field of tissue regeneration.

References

Allen CW (1981) Organofluorophsphazenes. A short review. Ind Eng Chem Prod Res Dev 20:77–79

Andrianov AK (2009) Polyphosphazenes for biomedical applications. Wiley, Hoboken

Barker SA, Young NM (1966) Isolation of hyaluronic acid by gel filtration on agarose. Carbohydr Res 2:363–370

Behl M, Lendlein A (2007) Actively moving polymers. Soft Matter 3:58–67

Bera S, Jedlinski Z (1993) Block segmented polymers – a new method of synthesis of copoly(amide-ester) ester polymer. J Polym Sci A Polym Chem 31:731–739

Boas NF (1949) Isolation of hyaluronic acid from the cocks comb. J Biol Chem 181:573–575

Brannon-Peppas L (1997) Polymers in controlled drug delivery. Med Plast Biomat 4:34–45

Bucher JE, Slade WC (1909) The anhydrides of isophthalic and terephthalic acids. J Am Chem Soc 31:1319–1321

Cardy RH (1979) Carcinogenicity and chronic toxicity of 2,4-toluenediamine in f344 rats. J Natl Cancer Inst 62:1107–1116

Chabot F et al (1983) Configurational structures of lactic-acid stereocopolymers as determined by ^{13}C-labeled ^{1}H-NMR. Polymer 24:53–59

Commandeur S, van Beusekom HM, van der Giessen WJ (2006) Polymers, drug release, and drug-eluting stents. J Interv Cardiol 19(6):500–506

Crivello JV et al (1996) Ketene acetal monomers: synthesis and characterization. J Polym Sci A Polym Chem 34:3091–3102

Deasy PB et al (1989) Preparation and characterization of lactic glycolic acid polymers and copolymers. J Microencapsul 6:369–378

Deng M, Nair LS, Nukavarapu SP, Kumbar SG, Jiang T, Weikel AL, Krogman NR, Allcock HR, Laurencin CT (2010) In situ porous structures: a unique polymer erosion mechanism in biodegradable dipeptide-based polyphosphazene and polyester blends producing matrices for regenerative engineering. Adv Funct Mater 20(17):2743–2957

Di Lullo GA et al (2002) Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. J Biol Chem 277:4223–4231

Domb AJ et al (1994) Polyanhydrides as carriers of drugs. In: Shalaby SW (ed) Biomedical polymers. Hanser, Munich, pp 69–96

Dorri M, Nasser M, Oliver R (2009) Resorbable versus titanium plates for facial fractures. Cochrane Database Syst Rev 21(1):CD007158

Feng YK, Guo JT (2009) Biodegradable polydepsipeptides. Int J Mol Sci 10:589–615

Friedlaender GE et al (2001) Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions: a prospective, randomized clinical trial comparing rhop-1 with fresh bone autograft. J Bone Joint Surg 83:S151

Govender S et al (2002) Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures – a prospective, controlled, randomized study of four hundred and fifty patients. J Bone Joint Surg 84A:2123–2134

Greenstein G, Caton JG (1993) Biodegradable barriers and guided tissue regeneration. Periodontol 2000 2000(1):36–45

Grigat E et al (1998) Bak 1095 and bak 2195: completely biodegradable synthetic thermoplastics. Polym Degrad Stab 59:223–226

Gunatillake P et al (2006) Recent developments in biodegradable synthetic polymers. Biotechnol Annu Rev 12:301–347

Guo K, Chu CC (2007) Synthesis, characterization, and biodegradation of copolymers of unsaturated and saturated poly(ester amide)s. J Polym Sci A Polym Chem 45:1595–1606

Han MG et al (2008) Synthesis and degradation behavior of poly(ethyl cyanoacrylate). Polym Degrad Stab 93:1243–1251

Heller J et al (1983) Controlled release of contraceptive steroids from biodegradable poly(ortho esters). Contracept Deliv Syst 4:43–53

Heller J et al (1992) Synthesis and characterization of a new family of poly(ortho ester)s. Macromolecules 25:3362–3364
Heller J et al (1995) Poly(ortho esters) for the pulsed and continuous delivery of peptides and proteins. In: Lee VHL et al (eds) Trends and future perspectives in peptide and protein drug delivery, vol 4. Harwood Academic Publ Gmbh, Chur, pp 39–56
Heller J et al (2002) Poly(ortho esters): synthesis, characterization, properties and uses. Adv Drug Deliv Rev 54:1015–1039
Hill JW, Carothers WH (1932) Studies of polymerization and ring formation. XIV. A linear superpolyanhydride and a cyclic dimeric anhydride from sebacic acid. J Am Chem Soc 54:1569–1579
Hodde J (2006) Extracellular matrix as a bioactive material for soft tissue reconstruction. ANZ J Surg 76:1096–1100
Hoffman AS (2002) Hydrogels for biomedical applications. Adv Drug Deliv Rev 54:3–12
Horton VL et al (1988) Comparison of bioabsorbable poly(ester-amide) monomers and polymers in vivo using radiolabeled homologs. In: Gebelijn CG, Dunn RL (eds) Progress in biomedical polymers. Plenum Press, New York, pp 263–282
Hughes CS, Postovit LM, Lajoie GA (2010) Matrigel: a complex protein mixture required for optimal growth of cell culture. Proteomics 10:1886–1890
Jain JP et al (2005) Role of polyanhydrides as localized drug carriers. J Control Release 103:541–563
Kaidar-Person O, Rosenthal RJ, Wexner SD, Szomstein S, Person B (2008) Compression anastomosis: history and clinical considerations. Am J Surg 195(6):818–826
Konan S, Haddad FS (2009) A clinical review of bioabsorbable interference screws and their adverse effects in anterior cruciate ligament reconstruction surgery. Knee 16:6–13
Kricheldorf HR, Serra A (1985) Polylactones. 6. Influence of various metal-salts on the optical purity of poly(l-lactide). Polym Bull 14:497–502
Kricheldorf HR, Stricker A (2000) Macrocycles. 13. Stannylenated glucose glycosides as cyclic initiators of epsilon-caprolactone and the synthesis of biodegradable networks. Macromolecules 33:696–701
Kroehne V et al (2008) Use of a novel collagen matrix with oriented pore structures for muscle cell differentiation in cell culture and in grafts. J Cell Mol Med 12:1640–1648
Krogman N, Singh A, Nair LS, Laurencin CT, Allcock HR (2006) The miscibility of bioerodible polyphosphazene/poly(lactide-co-glycolide) blends. Biomacromolecules 2:1–10
Kulkarni A et al (2007) Hydrolytic degradation of poly(*rac*-lactide) and poly[(*rac*-lactide)-co-glycolide] at the air-water interface. Surf Interface Anal 39:740–746
Kuppermann BD et al (2007) Dexamethasone DDS phase II study group. Randomized controlled study of an intravitreous dexamethasone drug delivery system in patients with persistent macular edema. Arch Ophthalmol 125(3):309–317
Lakshmi S, Katti DS, Laurencin CT (2003) Biodegradable polyphosphazenes for drug delivery applications. Adv Drug Deliv Rev 55(4):467–482
Laurencin CT et al (1995) In: Hollinger JO (ed) Biomedical applications of synthetic biodegradable polymers. CRC-Press, Boca Raton, pp 59–101
Leaper D, Assadian O, Hubner NO, McBain A, Barbolt T, Rothenburger S, Wilson P (2011) Antimicrobial sutures and prevention of surgical site infection: assessment of the safety of the antiseptic triclosan. Int Wound J 6:556–566
Leenslag JW, Pennings AJ (1987) Synthesis of high-molecular-weight poly(L-lactide) initiated with tin 2-ethylhexanoate. Makromol Chem Macromol Chem Phys 188:1809–1814
Leenslag JW et al (1984) Resorbable materials of poly(l-lactide). 5. Influence of secondary structure on the mechanical-properties and hydrolyzability of poly(L-lactide) fibers produced by a dry-spinning method. J Appl Polym Sci 29:2829–2842
Lendlein A (1999) Polymere als implantatwerkstoffe. Chem unserer Zeit 33:279–295
Lendlein A, Kelch S (2002) Shape-memory polymers. Angew Chem Int Ed 41:2034–2057
Lendlein A, Langer R (2002) Biodegradable, elastic shape-memory polymers for potential biomedical applications. Science 296:1673–1676

Lendlein A et al (1998) Tissue-compatible multiblock copolymers for medical applications, controllable in degradation rate and mechanical properties. Macromol Chem Phys 199:2785–2796
Li X, Jastri BR (2006) Biodegradable polymeric delivery systems. Design of controlled release drug delivery systems. McGraw-Hill, New York, pp 271–304
Li LC et al (2002) Polyanhydride implant for antibiotic delivery – from the bench to the clinic. Adv Drug Deliv Rev 54:963–986
Little U et al (2009) Accelerated degradation behaviour of poly(epsilon-caprolactone) via melt blending with poly(aspartic acid-co-lactide) (pal). Polym Degrad Stab 94:213–220
Martinez MB et al (1997) Hydrolytic degradation of poly(ester amides) derived from carbohydrates. Macromolecules 30:3197–3203
Meek MF, Coert JH (2008) US Food and Drug Administration/Conformit Europe-approved absorbable nerve conduits for chemical repair of peripheral and cranial nerves. Ann Plast Surg 60(4):466–472
Nair LS, Laurencin CT (2007) Biodegradable polymers as biomaterials. Prog Polym Sci 32:762–798
Ng SY et al (1997) Synthesis and erosion studies of self-catalyzed poly(ortho ester)s. Macromolecules 30:770–772
Nieuwenhuis J (1992) Synthesis of polylactides, polyglycolides and their copolymers. Clin Mater 10:59–67
Ormiston JA, Serruys PW (2009) Bioabsorbable coronary stents. Circ Cardiovasc Interv 2(3):255–260
Paredes N et al (1998) Synthesis and characterization of a family of biodegradable poly(ester amide)s derived from glycine. J Polym Sci A Polym Chem 36:1271–1282
Pasternak B, Rehn M, Andersen L et al (2008) Doxycycline-coated sutures improve mechanical strength of intestinal anastomoses. Int J Colorectal Dis 23:271–276
Peltoniemi H et al (2002) The use of bioabsorbable osteofixation devices in craniomaxillofacial surgery. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 94(1):5–14
Peppas NA (ed) (1987) Hydrogels in medicine and pharmacy. CRC Press, Boca Raton
Pietrzak WS, Eppley BL (2000) Resorbable polymer fixation for craniomaxillofacial surgery: development and engineering paradigms. J Craniofac Surg 11(6):575–585
Pillai CK, Sharma CP (2010) Review paper: absorbable polymeric surgical sutures: chemistry, production, properties, biodegradability, and performance. J Biomater Appl 25(4):291–366
Piskin E (1995) Biodegradable polymers as biomaterials. J Biomat Sci Polym Ed 6:775–795
Purcell DB et al (2004) Bioabsorbable interference screws in ACL reconstruction. Oper Tech Sports Med 12:180–187
Qiu Y, Park K (2001) Environment-sensitive hydrogels for drug delivery. Adv Drug Deliv Rev 53:321–339
Schmidmaier G, Lucke M, Wildemann B, Haas NP, Raschke M (2006) Prophylaxis and treatment of implant-related infections by antibiotic-coated implants: a review. Injury 37 (Suppl 2):S105–S112
Sethuraman S, Nair LS, El-Amin S, Nguyen MT, Singh S, Greish YE, Allcock HR, Brown PE, Laurencin CT (2011) Development and characterization of biodegradable nanocomposite injectables for orthopaedic applications. J Biomat Sci Polym Ed 22:733–752
Shalaby SW, Johnson A (1994) Synthetic absorbable polyesters. In: Shalaby SW (ed) Biomedical polymers: designed-to-degrade systems. Hanser, Munich, pp 1–34
Shih C et al (1993) Invivo and invitro release of ivermectin from poly(ortho ester) matrices. 1. Cross-linked matrix prepared from ketene acetal end-capped prepolymer. J Control Release 25:155–162
Spaans CJ et al (1998) High molecular weight polyurethanes and a polyurethane urea based on 1,4-butanediisocyanate. Polym Bull 41:131–138
Spotnitz WD, Burks S (2008) Hemostats, sealants, and adhesives: components of the surgical toolbox. Transfusion 48:1502–1516
Syzcher M (ed) (1999) Syzcher's handbook of polyurethanes. CRC Press, Boca Raton
Tang RP et al (2009) Poly(ortho ester amides): acid-labile temperature-responsive copolymers for potential biomedical applications. Biomacromolecules 10:722–727

The European Society for Biomaterials (1991) 2nd consensus conference on definitions in biomaterials 7–8th September. J Mater Sci Mater Med 2:62
Tsuji H et al (2003) Surface hydrophilicity and enzymatic hydrolyzability of biodegradable polyesters: 1. Effects of alkaline treatment. Polym Int 52:843–852
Ueda H, Tabata Y (2003) Polyhydroxyalkanoate derivatives in current clinical applications and trials. Adv Drug Deliv Rev 55(4):501–518
Vaccaro AR et al (2002) Bone grafting alternatives in spinal surgery. Spine J 2:206–215
Vera M et al (2006) Microspheres from new biodegradable poly(ester amide)s with different ratios of L- and D-alanine for controlled drug delivery. J Microencapsul 23:686–697
Vert M (1986) Biomedical polymers from chiral lactides and functional lactones – properties and applications. Makromol Chem Macromol Symp 6:109–122
Vert M (1989) Bioresorbable polymers for temporary therapeutic applications. Angew Makromol Chem 166:155–168
Weigel T et al (2006) Design and preparation of polymeric scaffolds for tissue engineering. Expert Rev Med Devices 3:835–851
Wolff LF, Mullally B (2000) New clinical materials and techniques in guided tissue regeneration. Int Dent J 50(5):235–244
Wu XS (1995) Synthesis and properties of biodegradable lactic/glycolic acid polymers. In: Wise DL, Altobelli DE, Yaszemski MJ, Gresser DJ, Schwartz ER (eds) Encyclopaedic handbook of biomaterials and bioengineering. Marcel Decker, New York, pp 1015–1054
Xiong X et al (2007) Isolated nature-identical collagen, WO 2007/137827 A1
Ye T et al (2008) Enhanced cell affinity of poly(l-lactide) film by immobilizing phosphonized chitosan. Appl Surf Sci 255:446–448

Chapter 22
Biomaterials-Enabled Regenerative Medicine in Corneal Applications

Naresh Polisetti, Christopher R. McLaughlin, Geeta K. Vemuganti, and May Griffith

Abstract The human cornea is the transparent outermost surface of the eye and the major refractive element of the visual system; its function depends on its optical clarity. Irreversible loss of optical quality of the cornea due to disease or damage results in permanent vision loss or blindness, necessitating a surgical replacement of the cornea (keratoplasty) in entirety or in part. While keratoplasty is considered one of the most successful forms of transplantation, lack of availability of donor tissues and rejection are major limiting factors. Advances in knowledge of biomaterials and stem cell biology have paved the way for tissue engineering of various organs including cornea. An ideal biomimetic for corneal tissue replacement would be the one which is transparent, provides mechanical support, promotes epithelial resurfacing, corneal innervation, and integrates into the surrounding corneo-scleral tissues and combats infection when challenged. This chapter reviews the advances made in developing various biomaterials for ocular application with or without cells.

N. Polisetti, Ph.D.
Department of Ophthalmology, Electron Microscopy and Cell Biology, University of Erlangen-Nuernberg, Schwabachanlage 6, Erlangen 91054, Germany
e-mail: naresh.polisetti@uk-erlangen.de

C.R. McLaughlin, Ph.D.
Michael G. DeGroote School of Medicine, McMaster University, Hamilton, ON 289-339-5288, Canada

G.K. Vemuganti, M.D., DNB, FAMS, FICP
School of Medical Sciences, University of Hyderabad, Prof C R Rao Road, Gachibowli, Hyderabad 500 046, Andhra Pradesh, India

M. Griffith, Ph.D., MBA (✉)
Integrative Regenerative Medicine (IGEN) Centre, Department of Clinical and Experimental Medicne, Linköping University, Cell Biology Building, Level 10, Linköping 58185, Sweden
e-mail: may.griffith@liu.se

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_22,

22.1 Introduction

22.1.1 The Cornea

The cornea is the transparent, dome-shape front surface of the eye, and the main structure that focuses light entering the eye to the retina for vision. Hence, its transparency is critical. It is comprised of five layers, three cellular (epithelium, stroma, and endothelium), and two acellular (Bowman's layer or membrane, and Descemet's membrane) (Fig. 22.1a).

The outermost epithelial layer consists of 5–6 layers of stratified, non-keratinizing cells, representing about 10% of the total corneal thickness. It is responsible for protecting the eye against foreign material, including pathogens, as well as absorbing oxygen and nutrients from the tear film. Corneal integrity and function are dependent upon the self-renewing properties of the corneal epithelium, which is maintained by the presence of stem cells located in the limbus region, at the corneaconjunctiva interface (Fig. 22.1b). These stem cells, known as limbal stem cells (LSC) or limbal epithelial stem cells (LESC) can divide both symmetrically to self-renew, and asymmetrically to produce daughter transiently amplifying cells (TAC) that migrate centripetally to populate the basal layer of the corneal epithelium (Kinoshita et al. 1981). The TAC divides and migrates superficially, progressively becoming more differentiated, and eventually becoming post-mitotic terminally differentiated cells.

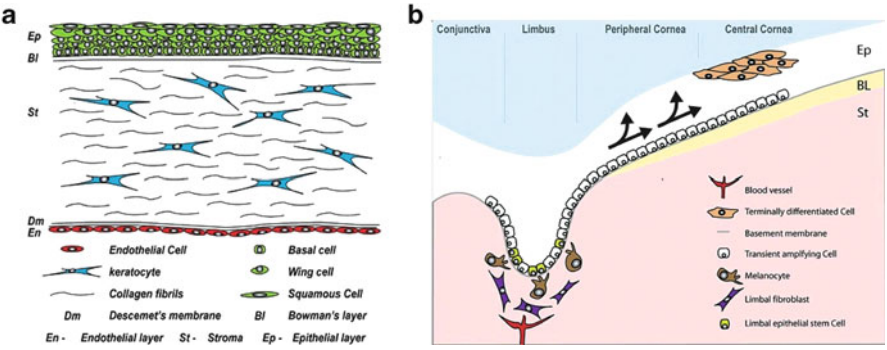


Fig. 22.1 (**a**) Cross-section through the human cornea. The outer surface comprises an epithelial layer that rests on a basement membrane, which in turn overlies a cell-free layer: Bowman's membrane. The middle cellular stromal layer contains a mainly collagenous extracellular matrix sparsely populated with keratocytes. The innermost layer consists of a single sheet of endothelial cells, which is physically separated from the stroma by the acellular Descemet's membrane. (**b**) Diagram of the human limbus. Limbal epithelial stem cells reside in the basal layer of the epithelium (*Ep*), at the limbus where the peripheral cornea meets the conjunctivum. Daughter transient amplifying cells divide and migrate towards the central cornea (*arrows*) to replenish the epithelium, which rests on Bowman's layer (*BL*). The stroma (*St*), of the limbal epithelial stem cell niche is vascularized and, is populated with fibroblasts and melanocytes (From: Secker and Daniels 2009)

Underlying the epithelium is the stroma. In adults, the stroma is approximately 500 μm thick and is composed of flatterned, interconnected cells called keratocytes embedded in an extracellular matrix (ECM) (Poole et al. 1996) of hydrated type I/V heterotypic collagen fibrils (15% wet weight) of uniform diameter (32 nm) in humans (Meek and Leonard 1993). Interspersed amongst the fibrillar collagens glycosoaminoglycans (GAGs) and proteoglycans (Axelsson and Heinegard 1975), such as keratin sulfate and dermatan sulfate (1% wet weight Anseth 1961); and other protein constituents including fibronectin, laminin, and type VI collagen. The collagen fibrils are packed in 300–500 parallel arrays (lamellae) tangential to the corneal surface (Hamada et al. 1972), that are principally responsible for the tensile mechanical properties of the cornea. The proteoglycans and their associated GAGs contribute to the corneas compressive and swelling material properties (Hedblom 1961), and to the uniform spacing of the collagen fibrils. The stromal cells or keratocytes are relatively quiescent in the healthy, uninjured cornea.

The innermost layer comprises a single layered endothelium, which is essential for the maintenance of appropriate stromal hydration for corneal transparency. Corneal endothelial cells contain Na^+/K^+ ATPase pumps that circulate aqueous humor between the anterior chamber and stroma (Nishida et al. 1997).

Bowman's layer (BL) is a cell-free, non-regenerating layer located between the epithelial basement membrane and the anterior corneal stroma of human corneas (Kenyon 1983). It is approximately 8–14 μm thick, and is comprised mainly of randomly oriented type I and V collagen fibrils (Hogan et al. 1971). It has a smooth anterior surface that underlies the epithelial basement membrane, and a posterior surface that merges with the less dense, but ordered, collagen lamellae of the corneal stroma proper. Unmyelinated nerve axons penetrate BL to terminate within the epithelium (Klyce and Beuerman 1988). The functional role of BL is unknown, but it has been suggested that it may be superfluous to human corneal function (Wilson and Hong 2000).

Descemet's membrane is the second acellular layer, and is essentially a thickened basement membrane, with a unique structure that lies between stroma and the endothelial layer of cornea. It contains Type IV collagen and a significant amount of Type VIII collagen.

22.1.2 The Need for Alternatives to Donated Corneas for Transplantation

Diseases affecting the cornea are a major cause of blindness all over the world, second only to cataracts in overall importance (Whitcher et al. 2001). Using the World Health Organizations (WHO; Geneva, Switzerland) definition of blindness, it is estimated that number of people with visual impairment is 285 million, with 65% of individuals aged over 50 years. Of these, 246 million have low vision (63% over 50 years old) and 39 million are estimated to be blind (82% over 50 years old). Infectious conditions, trachoma and corneal ulcer, are common causes of vision loss

in the developing world, whereas non-infectious entities like corneal dystrophies and pseudophakic bullous keratopathy are more common in developed countries (Cosar et al. 2002; Edwards et al. 2002; Dada et al. 1999; Gupta et al. 2001).

Many corneal diseases are treatable by transplantation with donated corneal tissue, by penetrating keratoplasty, lamellar keratoplasty or endothelial keratoplasty. Corneal transplants are not always possible in many parts of the world including some developed countries (Muraine et al. 2002), due to limitations in the storage and distribution of corneal tissue, or because of cultural or religious barriers. Even with available donor tissues, the success rate for transplantation beyond the first few years is moderately low. For example, long term survival is about 60% at 10 years (Coster and Williams 2005). Moreover, the supply of human corneal tissue is expected to diminish further due to an aging population and corresponding need for transplantation, and the increasing popularity of refractive surgery such as laser-assisted in situ keratomileusis (LASIK), which renders these corneas unacceptable for donation and with the increasing incidence of infectious diseases, including Acquired Immunodeficiency Syndrome (AIDS), hepatitis and Herpes Simplex Keratitis (Trinkaus-Randall 2000; Khan et al. 2001).

In addition to the short supply of donor corneas, an additional serious disadvantage of cornea allograft transplantation is the potential possibility for transmission of infection, as mentioned above. Hence, all donated corneas are screened at very high costs, as person-to-person transmission of the rabies virus (Houff et al. 1979) and at least one case of Creutzfeldt-Jakob disease (Duffy et al. 1974) have been reported. Even though very rare another concern is that transmission of as yet unknown pathogens could also occur.

There are also conditions that are not amenable to donor allograft transplantation. These include autoimmune conditions or cases where the ocular surface is badly damaged by disease or injury. For example, pathologies that chronically disrupt the ocular surface mucosa (ocular cicatricial pemphigoid, Stevens-Johnson syndrome, etc.) or disrupt tear production (Sjogren's) or injuries (severe chemical or alkali burns) that destroy the limbal stem cell niches (Limbal Stem Cell Deficiency; LSCD) are contraindications for donor grafts because the ocular surface will not re-epithelize properly and is prone to continuous de-epithelization.

Hence, there have been significant efforts in the development of both biomaterials and stem cell based methods and combinations of both, to replace part or the full thickness of damaged or diseased corneas. The best known alternatives to human allograft tissue are the "Artificial Corneas" which refer to corneal prostheses or keratoprostheses (KPro's). The classical KPro's were developed using plastic-based materials and were designed to restore minimal light transmission and protective functions of the cornea (Wilhelmus et al. 1995; Carlsson et al. 2003). These devices have now been used clinically but only as last resorts to save corneal vision, as they are still associated with *in vivo* complications like retroprosthetic membrane formation, infection and glaucoma.

There are a several reviews that cover the traditional KPro's (Myung et al. 2008; Gomaa et al. 2010; Rafat et al. 2010). Hence, in this chapter, we focus on biomaterials-enabled corneal regeneration and the various technologies developed in this area. These include the newer KPro's that have biointeractive functionality to enable

overgrowth of corneal epithelial cells for better integration, to the use of biointeractive, biodegradable implants that induce regeneration of corneal tissues and those used in stem cell delivery in cell based therapies for corneal diseases.

22.2 Keratoprostheses Enhanced for Biointeraction and Regeneration

KPro's epithelization has been argued to be important for the reduction of post-operative complications-infection in particular by restoring the eyes natural cellular barrier to external contaminants. Therefore, biointeractive capability is now being incorporated into new KPro's design that will support for epithelial cell growth on the anterior surface of the device.

For epithelization to occur, two innovations are required: (1) a surface that supports the adhesion of cells, and (2) sufficient capacity for transporting solutes by bulk diffusion to adherent cells. It is not sufficient to have a surface that is just adhesive to cells; permeability to nutrients, primarily glucose, is required to maintain the health of an overlying epithelium. Permeability has been made possible by development materials include ionic copolymers of poly(2-hydroxyethyl methacrylate) (PHEMA), intrinsically higher water content homopolymers like poly(vinyl alcohol) (PVA) (Miyashita et al. 2006; Shimmura et al. 2003; Uchino et al. 2007) and hydrophilic double polymer networks of polyethylene glycol (PEG) and poly(acrylic acid) (PAA) (Myung et al. 2007, 2008). Myung and coworkers reported collagen-coupled poly(ethylene glycol)/poly (acrylic acid) (PEG/PAA) interpenetrating polymer networks allowed for epithelial coverage in wound healing models both *in vitro* and *in vivo* in rabbits (Myung et al. 2009). Recently, Karkhaneh and coworkers reported 2-hydroxy methacrylate acid polydimethylsiloxane (PDMS) films that were modified by oxygen plasma treatment, after which type I collagen was immobilized onto this modified surface (Karkhaneh et al. 2011). The authors showed the attachment and proliferation of epithelial cells onto the modified PDMS. Wang and coworkers reported that coating polymethyl methacrylate (PMMA) discs, the principal component of Boston KPro's, with hydroxyapatite greatly improved cell viability, implant adhesion to tissue, and biocompatibility compared with unmodified PMMA (Wang et al. 2011).

The adhesion of epithelial cells to KPro's can therefore be enhanced by modifying these materials using extracellular matrix proteins, cell adhesion peptides and various growth factors.

22.2.1 Extracellular Matrix Proteins and Cell Adhesion Peptides

Various investigators report improved epithelialization of polymers by coating with extracellular matrix (ECM) proteins, including collagen, laminin, and fibronectin, which mimic the epithelial basement membrane and promote cell adhesion and

migration such as in the wound healing process (Griffith et al. 2002; Sweeney et al. 2003; Evans et al. 2000). It is thought that matrix proteins present on an implant surface may trigger migrating cells to reform a basement membrane by ECM protein secretion and formation of adhesion complexes at the surfaces (Sweeney et al. 2003). Other studies have reported the covalent tethering of cell adhesion peptides such as IKVAV, YIGSR & RGD, (Kobayashi and Ikada 1991; Merrett et al. 2001; Aucoin et al. 2002; Wallace et al. 2005; Jacob et al. 2005) to the surface may further improve the epithelial cell adhesion versus simple adsorption of ECM proteins. Tanihara group have shown using collagen-like polypeptide poly(Pro-Hyp-Gly) conjugated with GRGDS and PHSRN peptides enhances cell adhesion, migration, stratification and proposed that these may be useful scaffold for tissue regeneration (Shibasaki et al. 2011).

22.2.2 Growth Factors

Using of growth factors is another strategy to improve epithelialization. Epidermal growth factor (EGF) is a potent stimulator of corneal epithelial cell proliferation, migration, and is active in the wound healing process. Covalent grafting of EGF onto various supports such as glass (Kuhl and Griffith-Cima 1996) or PDMS (Klenkler et al. 2005) substrates through the use of PEG linkers has been reported to have clear effects on cell growth, while adsorbed EGF showed no biological activity (Kuhl and Griffith-Cima 1996). Nontheless, covalent grafting combined with the use of PEG linker presents several ceveats. Boucher and coworkers tethered EGF via coiled coil interactions and showed enhanced adhesion, spreading and proliferation of human corneal epithelial cells compared to EGF that was either physically adsorbed or present in solution (Boucher et al. 2010).

22.2.3 Biomaterials with Anti-microbial Properties

Along with biocompatibility and biointegration, there is also a great need for keratoprostheses and other implantable medical devices that inherently resist bacterial infections long-term. Recently, non-leaching, long chained hydrophobic polycations that can be attached covalently to the material surface and render them strongly antimicrobial have been developed (Lewis and Klibanov 2005; Klibanov 2007). Specifically, immobilized N,N-hexyl, methyl-polyethylenimine (HMPEI) has broad antibacterial, antifungal, and antiviral properties (Milovic et al. 2005; Lin et al. 2002; Haldar et al. 2006). Behlau and coworkers covalently attached HMPEI to Boston KPros and showed an inhibitory effect on biofilm formation by *Staphylococcus aureu*s clinical isolates (Behlau et al. 2011). They have also showed that there was

no toxicity or adverse effect with HMPEI-derivatized materials after intrastromal or anterior chamber implantation in rabbits *in vivo*.

22.3 Biointeractive Implants with Regenerative Functions

22.3.1 Biopolymeric and Biomimetic Scaffolds for Promoting Regeneration

Two dimensional growth of cells has been demonstrated on the surfaces of many synthetic polymers, but three-dimensional growth (ingrowth or encapsulation) of living cells has only been demonstrated in a few, fully synthetic polymers, particularly poly(ethylene) oxide, poly(propylene) oxide, and poly(N-isopropyl acrylamide) (PNiPAAm) (Lee and Mooney 2001; Hoffman 2002). For encapsulation of cells, many natural biopolymer hydrogels such as those based on alginate, fibrinogen-fibrin, chitosan, agarose, albumin, collagens, and their derivatives, are widely used. Of these, hydrogels of collagen type I, the predominant biopolymer in the human cornea, are particularly attractive as matrix replacement scaffolds, partially because of their strength at relatively low concentrations, resulting from the virtually rigid rod properties of the collagen type I triple helix (Amis et al. 1985). In addition, collagen brings the cell attachment motif arginine-glycine-glutamic acid (RGD) (Pierschbacher and Ruoslahti 1987). However, both the biodegradation resistance of collagen type I and the strength of hydrogels in general at low concentrations (10% wt/vol) need to be enhanced by chemical crosslinking (Hoffman 2002).

The authors have tested a range of biomaterials as corneal substitutes. We found that hybrid bio-synthetic hydrogels based on collagen, NIPAAm, acrylic acid and N-acryloxysuccinimide grafted with YIGSR peptide induced epithelial, stromal and nerve regeneration (Li et al. 2003). These implants emulated the corneal extracellular matrix by allowing for cell-matrix interaction in the restoration of functional structures including the generation of a basement membrane between the implant and overlying epithelium, stromal cell, and nerve axon ingrowth; potentiating differentiated cell state; and integration into the host tissue.

A more robust implant comprises interpenetrating networks of collagen and 2-methaacryloyloxyethyl phosphorylcholine (MPC) was also implanted into eyes in animal models (Liu et al. 2009; McLaughlin et al. 2010). They remain anchored within the host corneas and permitted regeneration of functional corneal neves as different, active nerve sub-types were recorded within the implant (Mclaughlin et al. 2010). Unlike, purely collagen implants, which enabled cell and nerve regeneration in human clinical trials (Fagerholm et al. 2009), the MPC –reinforced implants also show enzyme resistance *in vitro* (Liu et al. 2009). When implanted into alkali burnt rabbit corneas, the collagen-MPC implants, compared to collagen-only implants, were able to prevent neovascularization (Hackett et al. 2011).

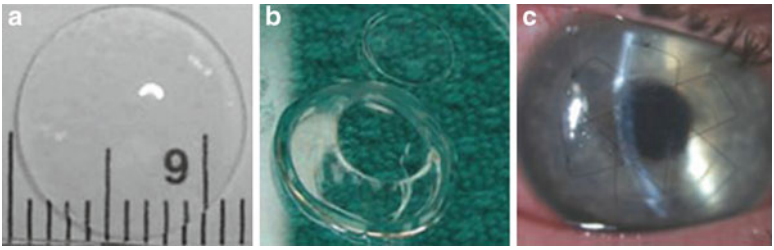


Fig. 22.2 Fabricated cornea and implantation method. (**a**) An example of optically clear, biosynthetic corneal substitutes used in these studies. (**b**) These were trephined to prepare a button for corneal implantation. Damaged host tissue was removed to a similar depth and diameter and replaced by this buttor. (**c**) After implantation, the button was held in place with three overlying 10–0 mattress sutures (Reproduced from: Fagerholm et al. 2010)

22.3.2 Biomimetic Tissue Engineered Corneal Substitute: First Clinical Study

In 2010, members of our current team tested ethyl(dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) crosslinked recombinant human collagen corneal substitutes (Fig. 22.2) in humans in a phase I clinical study as lamellar grafts in ten patients (Fagerholm et al. 2009). At two years post implantation, clinical results showed that implants are stably retained without adverse immune reactions. Six of the ten patients had improved vision (Fagerholm et al. 2010). Nine of the ten experienced corneal tissue, nerve and tear film regeneration, meaning that corneal epithelial cells grew over the implant, while stromal cell and nerves grew into the implant (Fig. 22.3), allowing for a tear film to form over the corneal surface. The tear film formation may have allowed the patients who were not able to tolerate contact lenses before to the surgery to be able to now wear contact lenses to improve their eyesight. However, long term monitoring and more extensive testing is needed to determine whether or not they will be useful as substitutes for donor corneal allograft tissue. In addition, further modifications, such as the use of interpenetrating networks, are likely needed to address the needs of a wider range of clinical indications such as full thickness implantation.

Fig. 22.3 (continued) of the unoperated cornea (**a**), regenerated corneal epithelial cells on the implant surface (**b**), and regenerated epithelium of the penetrating graft (**c**). Regenerated subbasal nerves (**e**) in an implanted cornea were parallel and morphologically similar to the normal cornea (**d**), whereas regenerated subbasal nerves were also observed in a cornea transplanted with human donor tissue (**f**). Anterior stromal cell (keratocyte) nuclei (**g–i**) and posterior keratocytes (**j–l**) were present, with varying density, in all corneas. The endothelium (**m–o**) in all corneas exhibited a characteristic mosaic pattern. Scale bars, 2 mm (OCT), 100 mm (IVCM) (Reproduced from: Fagerholm et al. 2010)

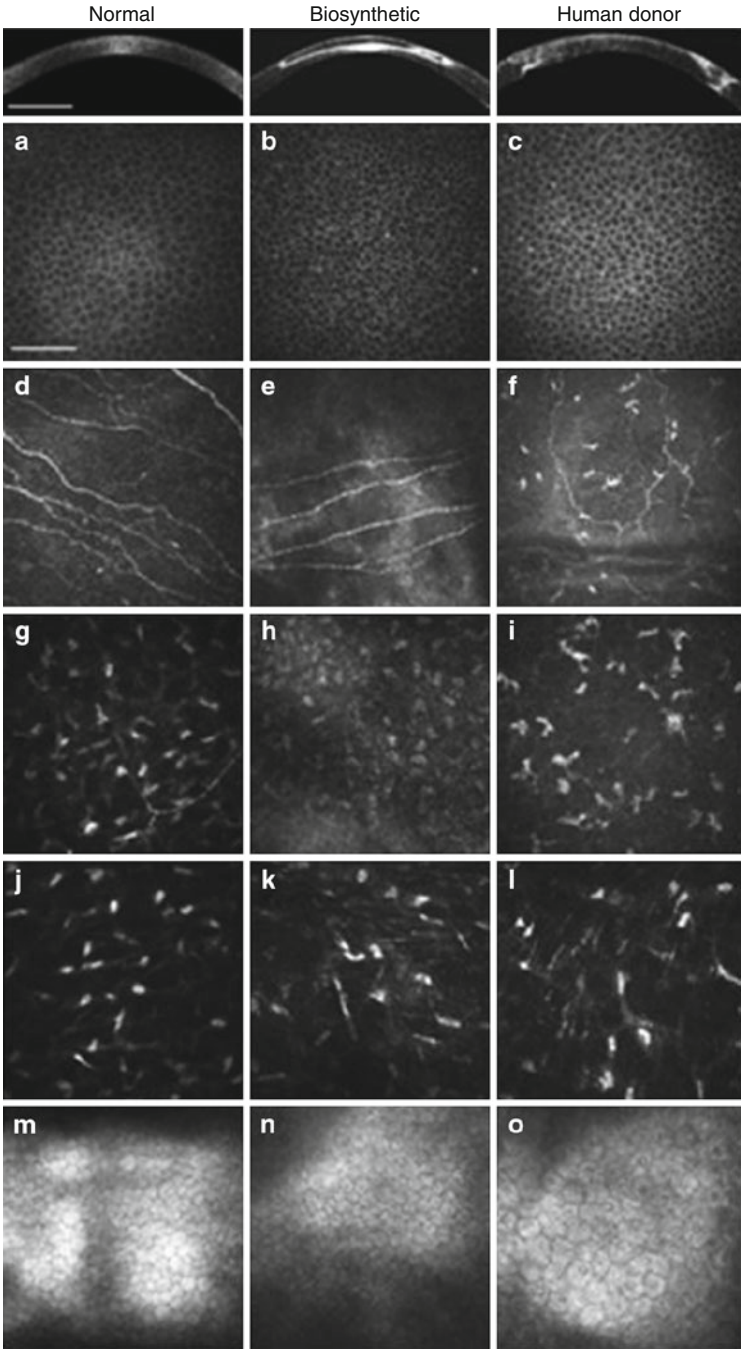


Fig. 22.3 Corneal features in a healthy, unoperated subject, alongside those of operated patients, at 24 months after implantation of a biosynthetic cornea or a human donor cornea. (*Top row*) Optical coherence tomography (OCT) images of a healthy cornea, biosynthetic implant, and human donor transplant by lamellar keratoplasty. Areas of wound-healing activity exhibit high reflectivity (*white areas*). (**a**–**o**) *In vivo* confocal microscopy (IVCM) images. Intact epithelium

22.4 Decellularized Corneas as Scaffolds

A recent development for preparing a corneal scaffold is to use decellularized tissue, from which the cells and antigen molecules are removed to diminish the host immune reaction. It has the same structure and composition as the natural tissue and it is thought that regeneration within the scaffold is regulated by donor cells. For decellularization of tissues, several groups have used detergents and enzymes and reported that the matrix structure of decellularized corneal stroma was preserved compared with that of native cornea (Ponce Marqvez et al. 2009; Choi et al. 2010; Gonzalez-Andrades et al. 2011).

To decellularize the cornea and to eliminate the toxic effect of chemicals, Hashimoto and coworkers (2010) have used high-hydrostatic pressurization (HHP) to decellularize porcine corneas and later transplanted these into rabbit corneas (Hashimoto et al. 2010). In the transplanted animals, no immune reaction occurred and turbid corneas became clear, showing that corneas obtained through HHP could be useful as scaffolds for tissue regeneration. Lee and coworkers (2011) demonstrated another method for decellularization of porcine cornea by freeze/thaw centrifugation with preservation of the corneal stroma (Lee et al. 2011)

Gonzalez-Andrades and coworkers (2011) showed that treatment of porcine corneas with 1.5 M sodium chloride treatment was able to generate an acellular corneal stroma with adequate histologic and optical properties. Human keratocytes were able to penetrate and spread within this scaffold with proper levels of cell differentiation (Gonzalez-Andrades et al. 2011). While these are promising results, the use of xenogeneic transplantation or use of poor quality cadaveric human corneas that cannot be used as living allografts will still require confirmation of safety, as immunogenicity and risk of disease transmissions are considerations that remain.

22.5 Biomaterials as Scaffolds in Cell-Based Therapies

22.5.1 Corneal Limbal Stem Cell Transplantation

22.5.1.1 Limbal Stem Cell Deficiency

In the previous section, regeneration of one or more corneal components such as the epithelium relied on the host or patient having a population of stem cells that enabled the reparative process. In several conditions, the patient's stem cell supply is depleted. This condition is referred as limbal epithelial stem cell deficiency (LSCD). Limbal stem cell deficiency can be primary, related to an insufficient stromal microenvironment to support stem cell function, such as aniridia, congential erythrokerato-dermia, keratitis associated with multiple endocrine deficiencies, neurotropic (neural and sichaemic) keratopathy and chronic limbitis; or secondary (more common) related to external factors that destroy limbal stem cells such as chemical (most common) or thermal injuries, Stevens-Johnson syndrome, pterygium

or severe microbial infections (Burman and Sangwan 2008). In these situations, persistent ulceration could occur, or, the conjunctival epithelial cells could move centrally to cover the depleted corneal surface. The re-epithelialization of the corneal surface by the conjunctival epithelium is accompanied with chronic inflammation, stromal scarring, neovascularization and persistent epithelial defects. Limbal stem cell deficiency can be diffuse (total) or sectoral (partial). In the latter case conjunctivalization of the corneal epithelium affects only part of the corneal surface.

22.5.1.2 Management of LSCD

There are various strategies for treating limbal stem cell deficiency. Partial limbal stem cells deficiency can be treated by mechanical debridment, and amniotic membrane transplantation (Tseng et al. 1998). In patients with total limbal stem cell deficiency, limbal auto-or allo- transplantation is indicated for corneal surface reconstruction. This may be combined with or followed by keratoplasty.

Drs. Kenneth Kenyon and Scheffer Tseng in (1989) were the first individuals to pioneer the use of human corneal limbal epithelial stem cells for treatment of ocular surface disorders (Tseng 1989; Kenyon and Tseng 1989). Several modifications have been described for this technique. In 1997, Pelligrini and co-workers produced cultured sheets of epithelium from a 1 mm^2 biopsy of healthy autologous limbal tissue and was transplanted onto the patient's limbal deficient eye. Two years post treatment, both patients still possessed stable corneal epithelium, an absence of vascularization, improved visual acuity and improvements in the subjective parameters of pain and photophobia (Pellegrini et al. 1997). Since then, many other groups have reported transplantation of corneal limbal epithelial stem cells to treat ocular surface disorders (Schwab et al. 2000; Tsai et al. 2000; Nakamura et al. 2004a).

Although all the techniques used in stem cell transplantation are similar in principle, the source of donor stem cells can be varying. Donor tissue can be obtained from the contralateral eye (limbal autograft) in cases of unilateral disease, or from consanguineous living donor (living HLA-matched donor) or from a cadaver donor (limbal allograft) when both eyes are affected (in bilateral conditions). The results (Shortt et al. 2007) have been quite promising in terms of improvement in vision. However, the mechanism of therapy efficacy remains unknown as there is no evidence of long-term donor allograft tissue survival. The disadvantage of this technique is that the patient is required to undergo systemic immunosuppression, and even then, the donor cells do not survive over the long term. This has led to the search of other autologous, non-corneal cell sources (Daya et al. 2005)

Several studies (Nakamura et al. 2004b, 2007; Inatomi et al. 2006a, b; Ang et al. 2006) demonstrated the efficacy of autologous cultivated oral mucosal epithelial transplantation (COMET) for the treatment of severe ocular surface disorders. Even though initial clinical results of COMET have been reported from several groups worldwide (Nishida et al. 2004b; Satake et al. 2008) the long-term clinical assessments of COMET are entirely unknown and feasibility of this technique still requires detailed investigation. A recent study by the Nakamura group have shown long term clinical results of COMET study are promising with improved visual acuity in ten

Table 22.1 Examples of different substrates used as carriers for expansion and transplantation of corneal limbal epithelial stem cells

	Substrate	Reference	Application
1	Human amniotic membrane	Schwab et al. (2000)	Clinical
		Tsai et al. (2000)	Clinical
		Sangwan et al. (2003)	Clinical
		Wang et al. (2003)	Research
2	Fibrin	Rama et al. (2001)	Clinical
		Talbot et al. (2006)	Research
3	Myogel	Francis et al. (2009)	Research
4	Soft contact lens	Deshpande et al. (2009)	Research
		Di Girolamo et al. (2009)	Clinical
5	Recombinant human collagen hydrogel	Dravida et al. (2008)	Research
6	Corneal stroma	Espana et al. (2003)	Research
7	Culture inserts	Koizumi et al. (2002)	Research
9	Silk fibroin	Lawrence et al. (2009)	Research
		Gil et al. (2010)	Research
		Chirila et al. (2008)	Research
10	Coated plates (Collagen IV, laminin, fibronectin)	Nakagawa et al. (1990)	Research
		Schwab et al. (2000)	Clinical
		Li et al. (2005)	Research
11	Collagne vitrigel membrane	Levis et al. (2010)	Research
		Takezawa et al. (2011)	Research
		McIntosh Ambrose et al. (2009)	Research
12	Temperature responsive polymers – Poly (N-Isopropylacrylamide)	Nishida et al. (2004a)	Research
		Sitalakshmi et al. (2009)	Research

eyes (53%) at postoperative 36 month (Nakamura et al. 2011). Other proposed sources of stem cells include buccal epithelial stem cells (Priya et al. 2011), hair follicle bulge derived stem cells (Meyer-Blazejewska et al. 2010) and umbilical mesenchymal cells (Liu et al. 2010).

Carrier tissue is needed in limbal transplants because it is not possible to transfer limbal stem cells alone. A range of substrates or biomaterials have been tested (Table 22.1) and several of these are described in more detail below.

Human Amniotic Membrane

The amniotic membrane is the innermost avascular layer of placenta and protects the embryo during gestation (Shrott et al. 2009). The first application of human amniotic membrane (HAM) in ophthalmology was in the successful treatment of a chemical burn of the ocular surface (de Roth 1940). After that, there are various reports for the ocular use of HAM highlighting an increasing number of new clinical indications and therapeutic applications. The structural integrity, transparency and elasticity of basement membrane of HAM make it the most widely accepted substrate for ocular surface reconstruction. Essentially, when stripped of its cell population,

that HAM acts as a decellularized scaffold. Various groups have shown that HAM has additional anti-inflammatory, anti-scarring and anti-angiogenic properties (Gomes et al. 2005; Hao et al. 2000; Tseng et al. 1999). HAM also produces growth factors such as epidermal growth factor (EGF), transforming growth factor α (TGF α), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and TGF-β that can stimulates epithelialization (Tosi et al. 2005; Koizumi et al. 2000). HAM can also act as a basement membrane that enables the migration of cells through the presence of laminin isoforms (Dua et al. 2004) and is therefore extensively used in ophthalmic surgery for corneal and conjunctival reconstruction (Fig. 22.4) (Dua et al. 2004).

Despite the successful use of HAM, however, caution is required as there is always an associated risk of disease transmission through the use of donated human tissue.

Temperature Responsive Corneal Cell Sheets

Okano and coworkers (Nishida et al. 2004a) developed an ingenious culture system based on a synthetic polymer surface that allows for carrier-free sheets of corneal epithelium to be cultured for transplantation. Temperature-responsive polymers chemically immobilized as thin films on cell culture surfaces facilitate cell adhesion and growth of cells in normal culture conditions at 37° C. They can reversibly alter their hydration properties with temperature change (hydrate and swell below 30° C) promoting complete detachment of adherent cells without the use of proteolytic enzymes or treatment with EDTA. They have developed human or rabbit corneal epithelial cell sheets using a novel temperature –responsive culture surface (Nishida et al. 2004a).

Mebiol gel, a copolymer comprised of thermoresponsive polymer poly(N-isopropylacrylamide-co-n-butyl methacrylate) (poly-NIPAAm-co- BMA) and hydrophilic polymer polyethylene glycol (PEG), is hydrophilic below 20 °C and hydrophobic above 20° C forming cross-linking points and a homogenous three-dimensional network in water (Vemuganti et al. 2009). This hydrogel has increased transparency compared to HAM (Vemuganti et al. 2009). Various studies on Mebiol gel shown, good proliferative capacity and exhibiting limbal and epithelial phenotype (Sudha et al. 2006) without any cytotoxicity (Madhavan et al. 2004). Transplantation of LSCs cultured on Mebiol gel showed that these cells may restore a nearly normal ocular epithelial surface in rabbit eyes with unilateral LSCD (Sitalakshmi et al. 2009).

The Cultured Autologous Oral Mucosal Epithelial Cell-Sheet (CAOMECS) is manufactured using a novel temperature-responsive culture well, UpCell®-Insert (Cellseed Inc, Tokyo, Japan) (Yamato et al. 2001), and is harvested without proteolytic processing retaining cell to cell junctions as well as deposited extra-cellular matrix of the basal membrane of the sheet (Burillon et al. 2011). The transplantation of CAOMECS is proposed for the treatment of total bilateral LSCD patients with moderate or severe symptoms, for whom any other treatments are not applicable. The efficacy of CAOMECS transplantation has been suggested by the presence of epithelium replacement in a clinical study including four patients suffering from total bilateral LSCD with severe loss of vision (<1/10) in Japan (Nishida et al. 2004b). A recent study considered CAOMECS transplantation a successful

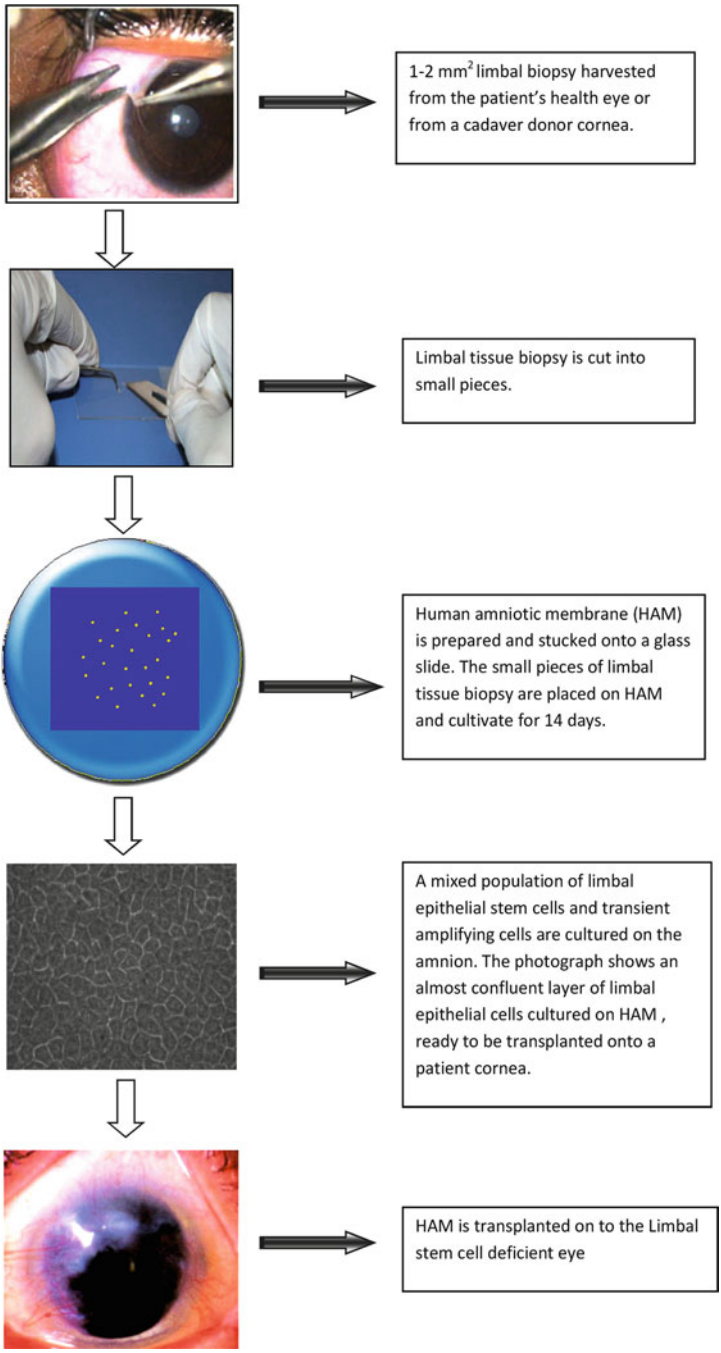


Fig. 22.4 Example of a limbal explant culture technique is used in clinical practice for treating patients with limbal stem cell deficiency

procedure, based on a composite criterion in 16 out of 25 patients assessed at 360 days post-graft (64%) (Burillon et al. 2011).

Soft Contact Lens

To develop an improved cell transfer system for delivering laboratory-cultured human limbal epithelial cells to the cornea, which would be low risk for the patient and convenient to use for the surgeons, researchers used contact lenses. In 2009, Deshpande and co-workers used acrylic acid polymers to coat the inner surface of a bandage contact lens for the delivery of limbal epithelial cells to patients. This methodology provides a culture surface, a transport vehicle and a method to immobilize the cells on the eye while protecting them when in position (Deshpande et al. 2009). Di Girolamo and coworkers cultured successfully human epithelial cells from a tissue explant on a non-coated contact lens and then transfer the cells onto the eye of three patients with LSCD to restore a transparent corneal epithelium (Di Girolamo et al. 2009).

Fibrin Sealant

A fibrin sealant or fibrin glue, used to create a fibrin clot, can be produced by combining fibrinogen and thrombin. Various groups have also used fibrin sealant as a substrate for LESC growth. It is particularly useful, as it is a readily biodegradable, natural substrate (Higa et al. 2007; Rama et al. 2001; Han et al. 2002). However, the use of fibrin gels may not be appropriate when a population of stem cells must be maintained, as it has been shown to affect cells by causing differentiation (Han et al. 2002).

Silk Fibroin

Silk fibroin membranes can be prepared from fibroin, a protein isolated from the domesticated silkworm (Bombyx mori) silk. It is a particularly useful material in corneal bioengineering as it is non-immunogenic while mechanically robust, transparent, easy to handle and has controlled degradation rates. Lawrence and coworkers have demonstrated that porous ultrathin fibroin films support the growth of primary rabbit corneal fibroblasts (Lawrence et al. 2009). Fibroblast growth on fibroin was slower than observed on tissue culture plastic, but the cells retained production of ECM molecules associated with a normal corneal phenotype. Nanopatterning technology allows surface modification of the silk fibroin with RGD peptide and this improved the alignment of corneal stromal cells and their growth (Gil et al. 2010).

Collagen

The major constituent of corneal stroma is collagen; therefore the use of collagen as substrate for corneal repair would be a good choice. Carbodiimide-crosslinked

recombinant human collagen type III has been tested as substrates and potential carriers for LESCs (Dravida et al. 2008). The thin hydrogels had a refractive index, transmission and backscatter properties that were similar to that of native cornea and LESCs were able to stratify and express putative stem cell and differentiated cell type markers in a similar fashion to cells on HAM. While crosslinking enhances the mechanical properties of collagen, major drawbacks could include the cytotoxicity of the crosslinker, reduced biomimetic qualities of the scaffold and prevention of cell based surface remodeling (Neel et al. 2006). Hence, crosslinker selection is important.

Collagen Vitrigel™ membrane has been reported to have superior optical properties as substrates. A vitrigel can be formed by three stage sequence: gelation, vitrification, and rehydration. Collagen vitrigel membrane composed of high density collagen fibrils equivalent to connective tissue in vivo and it possesses excellent transparency and permeability of protein with high molecular weight and consequently the various researchers utilizing it as a cell culture substratum (Takezawa et al. 2004). Takezawa and coworkers established an *in vitro* rabbit corneal epithelium model by culturing normal rabbit corneal epithelial cells on collagen vitrigel membranes and inducing differentiation to form a stratified epithelium (Takezawa et al. 2008). Cultured human limbal epithelial, bovine fibroblast and fabricated rabbit endothelial cells on vitrified collagen membranes showed both stem and differentiated phenotypes (McIntosh Ambrose et al. 2009). A recent study by the Takezawa group has used collagen vitrigel membrane for creating a corneal epithelial model for an ocular irritancy evaluation as an alternative to the Draize eye irritation test (Takezawa et al. 2011). LESCs grown on a plastic compressed collagen scaffolds showed a phenotype similar to that of central corneal cells (Levis et al. 2010)**.**

Various groups have produced electrospun collagen fibres from solutions that were combined with synthetic polymers (Buttafoco et al. 2006; Casper et al. 2007; Matthews et al. 2002; Zhong et al. 2006), but many of the polymers or solvents are cytotoxic and so not appropriate for use in cellular applications. Wray and Orwin group have produced collagen type I fibres using a less toxic solvent (Wray and Orwin 2009). They showed that corneal fibroblasts elongated along the axis of fibre alignment, responding changes in microstructure and organization of the matrix environment. This method appears to provide a viable scaffold material for corneal stroma replacement but again, further testing is needed to determine how LESCs would react to this material.

22.5.2 Corneal Endothelial Reconstruction

The use of cultured human corneal endothelial cells (HCECs) as an alternative to full thickness keratoplasty in the replacement of defective corneal endothelium was conceptualized over three decades ago (Jumblatt et al. 1978; Gospodarowicz et al. 1979). The *in vitro* proliferation capacity of HCECs is well established

(Joyce 2003; Baum et al. 1979; Koizumi et al. 2008; Proulx et al. 2009a) and their growth dynamics are now better understood (Joyce et al. 1996; Engelmann et al. 2004; Zhu and Joyce 2004). Various reports have shown the efficacy of vitrigel membrane (Takezawa et al. 2004; Koizumi et al. 2007, 2008), fibrin-agarose scaffolds (Alaminos et al. 2006), collagen chondroitin sulfate foams (Vrana et al. 2008), amniotic membrane (Ishino et al. 2004), gelatinious membranes (Lai et al. 2007; Hsiue et al. 2006; Sumide et al. 2006) as potential carrier systems for endothelial cells for transplantation purposes. The lifting of confluent HCECs cultured on a thermoresponsive polymeric culture surface as an intact HCEC sheet without the use of enzymatic dissociation has also been shown (Ide et al. 2006; Sumide et al. 2006).

Endothelium denuded corneal buttons have also been tested as carriers for corneal endothelial cells in *in vivo* models (Proulx et al. 2009a, b; Honda et al. 2009). The advantage of using such a carrier is that both normal corneal shape and corneal transparency is maintained. Tissue-engineered feline corneal endothelium using cultured feline CECs seeded onto devitalized cadaveric human stromal cornea achieved a cell density of $2{,}272 \pm 344$ cells/mm^2 and expressed characteristic function-related markers such as Na^+K^+-ATPase and ZO-1 (Proulx et al. 2009a). A subsequent report showed functional success when the tissue-engineered feline corneal endothelium, reconstructed on a devitalized human stromal carrier, was transplanted into a feline model (Proulx et al. 2009b). Promising results were obtained when HCECs-populated stromal discs was transplanted into rabbit models (Honda et al. 2009). Although promising, further refinement is needed prior to routined use of human corneal stromal buttons as a carriers in achieving clinically relevant endothelial cell density for transplantation. In addition, long-term functional assessment is needed. Furthermore, development of a defined serum free, xeno-free culture system for the expansion of HCECs will be required for future clinical trials (Peh et al. 2011).

22.6 Conclusion

There have been significant developments in regenerative medicine-based approaches to replace partial or the full thickness of damaged or diseased corneas. Biomaterials have been developed to assist in these reparative procedures. They have been designed as interactive scaffolds to promote endogenous stem cell repair and regeneration, and they have also been used as substrates for the implantation of exogenous stem cells. These different approaches in the near future be able to supplement the supply of human donor corneas harvested for transplantation. They may also be further developed to some day treat, diseased or damaged corneas that cannot be treated using currently available techniques. It should be noted that purely cell based techniques of injecting stem cells into damaged corneas are also being tested, but are not within the scope of this chapter.

References

Alaminos M, Del Carmen S-QM, Muñoz-Avila JI et al (2006) Construction of a complete rabbit cornea substitute using a fibrin-agarose scaffold. Invest Ophthalmol Vis Sci 47:3311

Amis EJ, Carriere CJ, Ferry JD et al (1985) Effect of pH on collagen flexibility determined from dilute solution viscoelastic measurements. Int J Biol Macromol 7:130–134

Ang LPK, Nakamura T, Inatomi T et al (2006) Autologous serum-derived cultivated oral epithelial transplants for severe ocular surface disease. Arch Ophthalmol 124:1543–1551

Anseth A (1961) Studies on corneal polysaccharides. III. Topographic and comparative biochemistry. Exp Eye Res 1:106–115

Aucoin L, Griffith CM, Pleizier G et al (2002) Interactions of corneal epithelial cells and surfaces modified with cell adhesion peptide combinations. J Biomater Sci Polym Ed 13:447–462

Axelsson I, Heinegard D (1975) Fractionation of proteoglycans from bovine corneal stroma. Biochem J 145:491–500

Baum JL, Niedra R, Davis C et al (1979) Mass culture of human corneal endothelial cells. Arch Ophthalmol 97:1136

Behlau I, Mukherjee K, Todani A et al (2011) Biocompatibility and biofilm inhibition of N, N-hexyl, methyl-polyethylenimine bonded to Boston Keratoprosthesis materials. Biomaterials 32(34):8783–8796

Boucher C, Ruiz JC, Thibault M et al (2010) Human corneal epithelial cell response to epidermal growth factor tethered via coiled-coil interactions. Biomaterials 31(27):7021–7031

Burillon C, Huot L, Justin V et al (2011) Cultured autologous oral mucosal epithelial cell-sheet (CAOMECS) transplantation for the treatment of corneal limbal epithelial stem cell deficiency. Invest Ophthalmol Vis Sci 55:1325–1331 [Epub ahead of print]

Burman S, Sangwan V (2008) Cultivated limbal stem cell transplantation for ocular surface reconstruction. Clin Opthalmol 2(3):489–502

Buttafoco L, Kolkman NG, Engbers-Buijtenhuijs P et al (2006) Electrospinning of collagen and elastin for tissue engineering applications. Biomaterials 27(5):724–734

Carlsson DJ, Li F, Shimmura S, Griffith M (2003) Bioengineered corneas: how close are we? Curr Opin Ophthalmol 14:192–197

Casper CL, Yang W, Farach-Carson MC et al (2007) Coating electrospun collagen and gelatin fibers with perlecan domain I for increased growth factor binding. Biomacromolecules 8(4):1116–1123

Chirila T, Barnard Z, Zainuddin Z et al (2008) Bombyx mori silk fibroin membranes as potential substrata for epithelial constructs used in the management of ocular surface disorders. Tissue Eng Part A 14:1203–1211

Choi JS, Williams JK, Greven M et al (2010) Bioengineering endothelialized neo-corneas using donor-derived corneal endothelial cells and decellularized corneal stroma. Biomaterials 31:6738–6745

Cosar CB, Sridhar MS, Cohen EJ et al (2002) Indications for penetrating keratoplasty and associated procedures, 1996–2000. Cornea 21:148–151

Coster DJ, Williams KA (2005) The impact of corneal allograft rejection on the long-term outcome of corneal transplantation. Am J Ophthalmol 140(6):1112–1122

Dada T, Sharma N, Vajpayee RB (1999) Indications for pediatric keratoplasty in India. Cornea 18:296–298

Daya SM, Watson A, Sharpe JR et al (2005) Outcomes and DNA analysis of ex vivo expanded stem cell allograft for ocular surface reconstruction. Ophthalmology 112:470–477

De Roth A (1940) Plastic repair of conjunctival defects with fetal membrane. Arch Ophthalmol 23:522–525

Deshpande P, Notara M, Bullett N et al (2009) Development of a surface-modified contact lens for the transfer of cultured limbal epithelial cells to the cornea for ocular surface diseases. Tissue Eng Part A 15(10):2889–2902

Di Girolamo N, Bosch M, Zamora K et al (2009) A contact lens-based technique for expansion and transplantation of autologous epithelial progenitors for ocular surface reconstruction. Transplantation 87(10):1571–1578
Dravida S, Gaddipati S, Griffith M et al (2008) A biomimetic scaffold for culturing limbal stem cells: a promising alternative for clinical transplantation. J Tissue Eng Regen Med 2(5):263–271
Dua HS, Gomes JAP, King AJ (2004) The amniotic membrane in ophthalmology. Surv Ophthalmol 49:51–77
Duffy P, Wolf J, Collins G et al (1974) Letter: possible person-to-person transmission of Creutzfeldt-Jakob disease. N Engl J Med 290:692–693
Edwards M, Clover GM, Brookes N et al (2002) Indications for corneal transplantation in New Zealand: 1991–1999. Cornea 21:152–155
Engelmann K, Bednarz J, Valtink M (2004) Prospects for endothelial transplantation. Exp Eye Res 78:573
Espana EM, Kawakita T, Romano A et al (2003) Stromal niche controls the plasticity of limbal and corneal epithelial differentiation in a rabbit model of recombined tissue. Invest Ophthalmol Vis Sci 44:5130–5135
Evans MD, Xie RZ, Fabbri M et al (2000) Epithelialization of a synthetic polymer in the feline cornea: a preliminary study. Invest Ophthalmol Vis Sci 41(7):1674–1680
Fagerholm P, Lagali NS, Carlsson DJ et al (2009) Corneal regeneration following implantation of a biomimetic tissue-engineered substitute. Clin Transl Sci 2:162–164
Fagerholm P, Lagali NS, Merrett K et al (2010) A biosynthetic alternative to human donor tissue for inducing corneal regeneration: 24-month follow-up of a phase 1 clinical study. Sci Transl Med 2:46ra61
Francis D, Abberton K, Thompson E, Daniell M (2009) Myogel supports the ex-vivo amplification of corneal epithelial cells. Exp Eye Res 88:339–346
Gil ES, Mandal BB, Park SH et al (2010) Helicoidal multi-lamellar features of RGD-functionalized silk biomaterials for cornealtissue engineering. Biomaterials 31(34):8953–8963
Gomaa A, Comyn O, Liu C (2010) Keratoprostheses in clinical practice – a review. Clin Experiment Ophthalmol 38:211–224
Gomes JAP, Romano A, Santos MS et al (2005) Amniotic membrane use in ophthalmology. Curr Opin Ophthalmol 16:233–240
Gonzalez-Andrades M, De La Cruz CJ, Ionescu AM et al (2011) Generation of bioengineered corneas with decellularized xenografts and human keratocytes. Invest Ophthalmol Vis Sci 52:215–222
Gospodarowicz D, Greenburg G, Alvarado J (1979) Transplantation of cultured bovine corneal endothelial cells to rabbit cornea: clinical implications for human studies. Proc Natl Acad Sci USA 76:464
Griffith M, Hakim M, Shimmura S et al (2002) Artificial human corneas: scaffolds for transplantation and host regeneration. Cornea 21(Suppl 2):S1–S8
Gupta V, Bambery P, Radotra BD et al (2001) Vogt-Koyanagi-Harada syndrome following injury-induced progressive vitiligo. Indian J Ophthalmol 49:53–55
Hackett JM, Lagali N, Merrett K et al (2011) Biosynthetic corneal implants for replacement of pathologic corneal tissue: performance in a controlled rabbit alkali burn model. Invest Ophthalmol 52(2):651–657
Haldar J, An D, Alvarez de Cienfuegos L et al (2006) Polymeric coatings that inactivate both influenza virus and pathogenic bacteria. Proc Natl Acad Sci USA 103(47):17667–17671
Hamada R, Giraud JP, Graf B et al (1972) Analytical and statistical study of the lamellae, keratocytes and collagen fibrils of the central region of the normal human cornea. (Light and electron microscopy). Arch Ophtalmol Rev Gen Ophtalmol 32:563–570
Han B, Schwab IR, Madsen TK et al (2002) A fibrin-based bioengineered ocular surface with human corneal epithelial stem cells. Cornea 21:505–510
Hao Y, Ma DH, Hwang DG et al (2000) Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. Cornea 19:348–352

Hashimoto Y, Funamoto S, Sasaki S et al (2010) Preparation and characterization of decellularized cornea using high-hydrostatic pressurization for corneal tissue engineering. Biomaterials 31:3941–3948

Hedblom EE (1961) The role of polysaccharides in corneal swelling. Exp Eye Res 1:81–91

Higa K, Shimmura S, Kato N et al (2007) Proliferation and differentiation of transplantable rabbit epithelial sheets engineered with or without an amniotic membrane carrier. Invest Ophthalmol Vis Sci 48:597–604

Hoffman AS (2002) Hydrogels for biomedical applications. Adv Drug Deliv Rev 54:3–12

Hogan MJ, Alvarado JA, Weddell JE (1971) Histology of the human eye. An atlas and textbook. Saunders, Philadelphia\London\Toronto

Honda N, Mimura T, Usui T et al (2009) Descemet stripping automated endothelial keratoplasty using cultured corneal endothelial cells in a rabbit model. Arch Ophthalmol 127:1321

Houff SA, Burton RC, Wilson RW et al (1979) Human-to-human transmission of rabies virus by corneal transplant. N Engl J Med 300:603–604

Hsiue GH, Lai JY, Chen KH et al (2006) A novel strategy for corneal endothelial reconstruction with a bioengineered cell sheet. Transplantation 81:473

Ide T, Nishida K, Yamato M et al (2006) Structural characterization of bioengineered human corneal endothelial cell sheets fabricated on temperature-responsive culture dishes. Biomaterials 27:607

Inatomi T, Nakamura T, Koizumi N et al (2006a) The mid-term results of ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation. Am J Ophthalmol 141:267–275

Inatomi T, Nakamura T, Kojyo M et al (2006b) Ocular surface reconstruction with combination of cultivated autologous oral mucosal epithelial transplantation and penetrating keratoplasty. Am J Ophthalmol 142:757–764

Ishino Y, Sano Y, Nakamura T et al (2004) Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. Invest Ophthalmol Vis Sci 45:800

Jacob JT, Rochefort JR, Bi J et al (2005) Corneal epithelial cell growth over tethered-protein/peptide surface-modified hydrogels. J Biomed Mater Res B Appl Biomater 72:198–205

Joyce NC (2003) Proliferative capacity of the corneal endothelium. Prog Retin Eye Res 22:359

Joyce NC, Meklir B, Joyce SJ et al (1996) Cell cycle protein expression and proliferative status in human corneal cells. Invest Ophthalmol Vis Sci 37:645

Jumblatt MM, Maurice DM, McCulley JP (1978) Transplantation of tissue-cultured corneal endothelium. Invest Ophthalmol Vis Sci 17:1135

Karkhaneh A, Mirzadeh H, Ghaffariyeh A et al (2011) Novel materials to enhance corneal epithelial cell migration on keratoprosthesis. Br J Ophthalmol 95(3):405–409

Kenyon KR (1983) Morphology and pathologic responses of the cornea to disease. Smolin and Thoft's the cornea: scientific foundations and clinical practice. Little, Brown & Co, Boston

Kenyon KR, Tseng SC (1989) Limbal autograft transplantation for ocular surface disorders. Ophthalmology 96:709–722, discussion 722–723

Khan B, Dudenhoefer EJ, Dohlman CH (2001) Keratoprosthesis: an update. Curr Opin Ophthalmol 12:282–287

Kinoshita JH, Kador P, Catiles M (1981) Aldose reductase in diabetic cataracts. JAMA 246:257–261

Klenkler BJ, Griffith M, Becerril C et al (2005) EGF-grafted PDMS surfaces in artificial cornea applications. Biomaterials 26:7286–7296

Klibanov AM (2007) Permanently microbicidal materials coatings. J Mater Chem 17:2479–2482

Klyce SD, Beuerman RW (1988) Structure and function of the cornea. In: Kaufman HE, Barron BA, McDonald MB, Waltman SR (eds) The cornea. Churchill Livingstone, New York

Kobayashi H, Ikada Y (1991) Corneal cell adhesion and proliferation on hydrogel sheets bound with cell-adhesive proteins. Curr Eye Res 10:899–908

Koizumi NJ, Inatomi TJ, Sotozono CJ et al (2000) Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res 20:173–177

Koizumi N, Cooper LJ, Fullwood NJ et al (2002) An evaluation of cultivated corneal limbal epithelial cells, using cell suspension culture. Invest Ophthalmol Vis Sci 43:2114–2121
Koizumi N, Sakamoto Y, Okumura N et al (2007) Cultivated corneal endothelial cell sheet transplantation in a primate model. Invest Ophthalmol Vis Sci 48:4519
Koizumi N, Sakamoto Y, Okumura N et al (2008) Cultivated corneal endothelial transplantation in a primate: possible future clinical application in corneal endothelial regenerative medicine. Cornea 27(suppl 1):S48
Kuhl PR, Griffith-Cima LG (1996) Tethered epidermal growth factor as a paradigm for growth factor-induced stimulation from the solid phase. Nat Med 2:1022–1027
Lai JY, Chen KH, Hsiue GH (2007) Tissue-engineered human corneal endothelial cell sheet transplantation in a rabbit model using functional biomaterials. Transplantation 84:1222
Lawrence BD, Marchant JK, Pindrus MA et al (2009) Silk film biomaterials for cornea tissue engineering. Biomaterials 30(7):1299–1308
Lee KY, Mooney DJ (2001) Hydrogels for tissue engineering. Chem Rev 101:1869–1879
Lee SH, Chun YS, Kim JC (2011) The study of characteristics of acellular porcine cornea using freezing-thawing-centrifugation. J Korean Ophthalmol Soc 52:86
Levis HJ, Brown RA, Daniels JT (2010) Plastic compressed collagen as a biomimetic substrate for human limbal epithelial cell culture. Biomaterials 31(30):7726–7737
Lewis K, Klibanov AM (2005) Surpassing nature: rational design of sterile-surface materials. Trends Biotechnol 23(7):343–348
Li F, Carlsson D, Lohmann C et al (2003) Cellular and nerve regeneration within a biosynthetic extracellular matrix for corneal transplantation. Proc Natl Acad Sci USA 100:15346–15351
Li DQ, Chen Z, Song XJ, de Paiva CS et al (2005) Partial enrichment of a population of human limbal epithelial cells with putative stem cell properties based on collagen type IV adhesiveness. Exp Eye Res 80:581–590
Lin J, Tiller JC, Lee SB et al (2002) Insights into bactericidal action of surface-attached poly(vinyl-N-hexylpyridinium) chains. Biotechnol Lett 24:801–805
Liu W, Deng C, Mclaughlin CR et al (2009) Collagen-phosphorylcholine interpenetrating network hydrogels as corneal substitutes. Biomaterials 30:1551–1559
Liu H, Zhang J, Liu C-Y et al (2010) Cell therapy of congenital corneal diseases with umbilical mesenchymal stem cells: lumican null mice. PLoS One 5(5):e10707
Madhavan HN, Malathi J, Joseph RP et al (2004) A study on the growth of continuous culture cell lines embedded in Mebiol Gel. Curr Sci 87:1275–1277
Matthews JA, Wnek GE, Simpson DG et al (2002) Electrospinning of collagen nanofibers. Biomacromolecules 3(2):232–238
McIntosh Ambrose W, Salahuddin A, So S et al (2009) Collagen Vitrigel membranes for the in vitro reconstruction of separate corneal epithelial, stromal, and endothelial cell layers. J Biomed Mater Res B Appl Biomater 90:818–831
Mclaughlin CR, Acosta MC, Luna C et al (2010) Regeneration of functional nerves within full thickness collagen-phosphorylcholine corneal substitute implants in guinea pigs. Biomaterials 31:2770–2778
Meek KM, Leonard DW (1993) Ultrastructure of the corneal stroma: a comparative study. Biophys J 64:273–280
Merrett K, Griffith CM, Deslandes Y et al (2001) Adhesion of corneal epithelial cells to cell adhesion peptide modified pHEMA surfaces. J Biomater Sci Polym Ed 12:647–671
Meyer-Blazejewska EA, Kruse FE, Bitterer K et al (2010) Preservation of the limbal stem cell phenotype by appropriate culture techniques. Invest Ophthalmol Vis Sci 51:765–774
Milovic NM, Wang J, Lewis K (2005) Immobilized N-alkylated polyethylenimine avidly kills bacteria by rupturing cell membranes with no resistance developed. Biotechnol Bioeng 90:715–722
Miyashita H, Shimmura S, Kobayashi H et al (2006) Collagen-immobilized poly(vinyl alcohol) as an artificial cornea scaffold that supports a stratified corneal epithelium. J Biomed Mater Res B Appl Biomater 76:56–63

Muraine MC, Collet A, Brasseur G (2002) Deep lamellar keratoplasty combined with cataract surgery. Arch Ophthalmol 120:812–815
Myung D, Koh W, Bakri A et al (2007) Design and fabrication of an artificial cornea based on a photolithographically patterned hydrogel construct. Biomed Microdevices 9:911–922
Myung D, Duhamel P-E, Cochran JR, Noolandi J et al (2008) Development of hydrogel-based keratoprostheses: a materials perspective. Biotechnol Prog 24:735–741
Myung D, Farooqui N, Zheng LL et al (2009) Bioactive interpenetrating polymer network hydrogels that support corneal epithelial wound healing. J Biomed Mater Res A 90:70–81
Nakagawa S, Nishida T, Kodama Y et al (1990) Spreading of cultured corneal epithelial cells on fibronectin and other extracellular matrices. Cornea 9:125–130
Nakamura T, Inatomi T, Sotozono C et al (2004a) Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. Br J Ophthalmol 88:1280–1284
Nakamura T, Inatomi T, Sotozono C et al (2004b) Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease. Acta Ophthalmol Scand 82:468–471
Nakamura T, Inatomi T, Cooper LJ et al (2007) Phenotypic investigation of human eyes with transplanted autologous cultivated oral mucosal epithelial sheets for severe ocular surface diseases. Ophthalmology 114:1080–1088
Nakamura T, Takeda K, Inatomi T et al (2011) Long-term results of autologous cultivated oral mucosal epithelial transplantation in the scar phase of severe ocular surface disorders. Br J Ophthalmol 95(7):942–946
Neel EAA, Cheema U, Knowles JC et al (2006) Use of multiple unconfined compression for control of collagen gel scaffold density and mechanical properties. Soft Matter 2:986–992
Nishida T, Nakamura M, Konma T et al (1997) Neurotrophic keratopathy–studies on substance P and the clinical significance of corneal sensation. Nippon Ganka Gakkai Zasshi 101:948–974
Nishida K, Yamato M, Hayashida Y et al (2004a) Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface. Transplantation 77:379–385
Nishida K, Yamato M, Hayashida Y et al (2004b) Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N Engl J Med 351:1187–1196
Peh GS, Beuerman RW, Colman A et al (2011) Human corneal endothelial cell expansion for corneal endothelium transplantation: an overview. Transplantation 91(8):811–819
Pellegrini G, Traverso CE, Franzi AT et al (1997) Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet 349:990–993
Pierschbacher MD, Ruoslahti E (1987) Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. J Biol Chem 262:17294–17298
Ponce Marquez S, Martinez VS, McInthosh Ambrose W et al (2009) Decellularization of bovine corneas for tissue engineering applications. Acta Biomater 5:1839–1847
Poole CA, Brookes NH, Clover GM (1996) Confocal imaging of the keratocyte network in porcine cornea using the fixable vital dye 5-chloromethylfluorescein diacetate. Curr Eye Res 15:165–174
Priya CG, Arpitha P, Vaishali S et al (2011) Adult human buccal epithelial stem cells: identification, ex-vivo expansion, and transplantation for corneal surface reconstruction. Eye (Lond) 25(12):1641–1649
Proulx S, Audet C, Uwamaliya J et al (2009a) Tissue engineering of feline corneal endothelium using a devitalized human cornea as carrier. Tissue Eng Part A 15:1709
Proulx S, Bensaoula T, Nada O et al (2009b) Transplantation of a tissue- engineered corneal endothelium reconstructed on a devitalized carrier in the feline model. Invest Ophthalmol Vis Sci 50:2686
Rafat MA, Hackett JM, Fagerholm P et al (2010) Artificial cornea. In: Dartt DA, Besharse J, Dana R (eds) Encyclopedia of the eye. Academic, Boston, USA, pp 128–134
Rama P, Bonni S, Lambiase A et al (2001) Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. Transplantation 72:1478–1485

Sangwan VS, Vemuganti GK, Iftekhar G et al (2003) Use of autologous cultured limbal and conjunctival epithelium in a patient with severe bilateral ocular surface disease induced by acid injury: a case report of unique application. Cornea 22:478–481
Satake Y, Dogru M, Yamane GY et al (2008) Barrier function and cytologic features of the ocular surface epithelium after autologous cultivated oral mucosal epithelial transplantation. Arch Ophthalmol 126:23–28
Schwab IR, Reyes M, Isseroff RR (2000) Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. Cornea 19:421–426
Secker GA, Daniels JT (2009) Limbal epithelial stem cells of the cornea. In: The Stem Cell Research Community (ed) StemBook. Harvard Stem Cell Institute: Cambridge, MA. Available from http://www.ncbi.nlm.nih.gov/books/NBK27054/. PubMed PMID: 20614614. Accessed 30 Jun 2009
Shibasaki Y, Hirohara S, Terada K et al (2011) Collagen-like polypeptide poly(Pro-Hyp-Gly) conjugated with Gly-Arg-Gly-Asp-Ser and Pro-His-Ser-Arg-Asn peptides enchances cell adhesion, migration, and stratification. Biopolymers 96(3):302–315
Shimmura S, Doillon CJ, Griffith M et al (2003) Collagen-poly(N-isopropylacrylamide)-based membranes for corneal stroma scaffolds. Cornea 22:S81–S88
Shortt AJ, Secker GA, Notara MD et al (2007) Transplantation of ex vivo cultured limbal epithelial stem cells: a review of techniques and clinical results. Surv Ophthalmol 52:483–502
Sitalakshmi G, Sudha B, Madhavan HN et al (2009) Ex vivo cultivation of corneal limbal epithelial cells in a thermoreversible polymer (Mebiol Gel) and their transplantation in rabbits: an animal model. Tissue Eng Part A 15(2):407–415
Sudha B, Madhavan HN, Sitalakshmi G et al (2006) Cultivation of human corneal limbal stem cells in Mebiol gel-A thermo-reversible gelation polymer. Indian J Med Res 124:655–664
Sumide T, Nishida K, Yamato M et al (2006) Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces. FASEB J 20:392
Sweeney DF, Xie RZ, Evans MDM et al (2003) A comparison of biological coatings for the promotion of corneal epithelialization of synthetic surface in vivo. Invest Ophthalmol Vis Sci 44:3301–3309
Takezawa T, Ozaki K, Nitani A et al (2004) Collagen vitrigel: a novel scaffold that can facilitate a three-dimensional culture for reconstructing organoids. Cell Transplant 13:463–473
Takezawa T, McIntosh-Ambrose W, Elisseeff JH (2008) A novel culture model of rabbit corneal epithelium utilizing a handy scaffold of collagen vitrigel membrane and its cryopreservation. Altern Anim Test Exp 13(Suppl):176
Takezawa T, Nishikawa K, Wang PC (2011) Development of a human corneal epithelium model utilizing a collagen vitrigel membrane and the changes of its barrier function induced by exposing eye irritant chemicals. Toxicol In Vitro 25(6):1237–1241
Talbot M, Carrier P, Giasson CJ, Deschambeault A, Guérin SL, Auger FA, Bazin R, Germain L (2006) Autologous transplantation of rabbit limbal epithelia cultured on fibrin gels for ocular surface reconstruction. Mol Vis 12:65–75
Tosi GM, Massaro-Giordano M, Caporossi A et al (2005) Amniotic membrane transplantation in ocular surface disorders. J Cell Physiol 202:849–851
Trinkaus-Randall V (2000) Cornea: biological responses. In: Lanza R, Langer R, Chick E (eds) Principles of tissue engineering, vol 2. Academic, San Diego, pp 471–491
Tsai RJ, Li LM, Chen JK (2000) Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. N Engl J Med 343:86–93
Tseng SC (1989) Concept and application of limbal stem cells. Eye (London, England) 3(Pt 2):141–157
Tseng SC, Prabhasawat P, Barton K et al (1998) Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. Arch Ophthalmol 116:431–441
Tseng SC, Li DQ, Ma X (1999) Suppression of transforming growth factor-beta isoforms, TGF-beta receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix. J Cell Physiol 179:325–335
Uchino Y, Shimmura S, Miyashita H, Uchino Y, Shimmura S, Miyashita H et al (2007) Amniotic membrane immobilized poly(vinyl alcohol) hybrid polymer as an artificial cornea scaffold that

supports a stratified and differentiated corneal epithelium. J Biomed Mater Res B Appl Biomater 81:201–206
Vemuganti GK, Fatima A, Madhira SL (2009) Limbal stem cells: application in ocular biomedicine. Int Rev Cell Mol Biol 275:133–181
Vrana NE, Builles N, Justin V et al (2008) Development of a reconstructed cornea from collagen-chondroitin sulfate foams and human cell cultures. Invest Ophthalmol Vis Sci 49:5325
Wallace C, Jacob JT, Stoltz A et al (2005) Corneal epithelial adhesion strength to tethered-protein/peptide modified hydrogel surfaces. J Biomed Mater Res A 72:19–24
Wang DY, Hsueh YJ, Yang VC et al (2003) Propagation and phenotypic preservation of rabbit limbal epithelial cells on amniotic membrane. Invest Ophthalmol Vis Sci 44:4698–4704
Wang L, Jeong KJ, Chiang HH et al (2011) Hydroxyapatite for keratoprosthesis biointegration. Invest Ophthalmol Vis Sci 52(10):7392–7399
Whitcher JP, Srinivasan M, Upadhyay MP (2001) Corneal blindness: a global perspective. Bull World Health Organ 79:214–221
Wilhelmus KR, Stulting RD, Sugar J et al (1995) Primary corneal graft failure. A national reporting system. Medical Advisory Board of the Eye Bank Association of America. Arch Ophthalmol 113:1497–1502
Wilson SE, Hong JW (2000) Bowman's layer structure and function: critical or dispensable to corneal function? A hypothesis. Cornea 19:417–420
Wray LS, Orwin EJ (2009) Recreating the microenvironment of the native cornea for tissue engineering applications. Tissue Eng Part A 15(7):1463–1472
Yamato M, Utsumi M, Kushida A et al (2001) Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature. Tissue Eng 7:473–480
Zhong S, Teo WE, Zhu X et al (2006) An aligned nanofibrous collagen scaffold by electrospinning and its effects on in vitro fibroblast culture. J Biomed Mater Res A 79(3):456–463
Zhu C, Joyce NC (2004) Proliferative response of corneal endothelial cells from young and older donors. Invest Ophthalmol Vis Sci 45:1743

Chapter 23
Functionalized Nanomaterials

Jie Zhou, Wenzhong Li, and Changyou Gao

Abstract Regenerative medicine aims to repair tissues or organs for restoring normal functions, which represents one of the greatest challenges in modern day science and medicine. Diverse techniques and materials are required to truly understand the process of tissue repairing and build a proper scaffold for cells attachment, proliferation and differentiation. Functionalized nanomaterials with nanotechnologies are the ideal to solve most of the problems of regenerative medicine. Multifunctionalized nanoparticles and nanostructured biomaterials can be powerful tools for cell tracking and matrix-like scaffold rebuilding

23.1 Introduction

Regenerative medicine is an interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneracy of cells, tissues or organs to restore impaired function resulting from any cause (Daar and Greenwood 2007),

J. Zhou, Ph.D.
R&D Center, Jushi Group Co., Ltd, 669 South Wenhua Road, Tongxiang Economic Development Zone, Tongxiang City, Zhejiang Province 314500, People's Republic of China

W. Li, Dr.-ing (NUS)
Center for Biomaterial Development and Berlin Brandenburg Center for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Kantstr. 55, Teltow 14513, Germany

C. Gao, Ph.D. (✉)
Department of Polymer Science and Engineering, Zhejiang University, No. 38, Zheda Road, Hangzhou, Zhejiang Province 310027, People's Republic of China
e-mail: cygao@zju.edu.cn

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_23,

which combines diverse techniques to stimulate or support the body's own self-healing capacity. Though numerous implants and engineered tissues have been developed which are based on the current knowledge of the superstructure and the microstructure of tissue in last several decades, the truly regenerative therapies require significant understanding and controlling the underlying nanostructures in cells, the extracellular matrix (ECM) and also the cell behaviors during the tissue regeneracy (Harrison 2008; Zhang and Webster 2009).

In order to truly understand of the regenerative process during the tissue reconstruction, the knowledge of cell migration, proliferation and/or differentiation after the regenerative therapies is critical. Cell imaging and cell tracking as one of these methods is used to investigate the detail process during tissue regeneracy. The knowledge from cell tracking helps to advance new technologies for improving regenerative medicine in vice versa (Solanki et al. 2008; Vaccaro et al. 2008). An ideal cell tracking technology should be multifunctional (able to be both imaged and quantified), high sensitive, nontoxic, have long life time after labeling, and label cells with high efficiency (Vaccaro et al. 2008).

Furthermore, in a regenerative strategy, a scaffold is normally required for promoting new tissue formation by providing adequate space (porosity) and appropriate surface to foster and direct cellular attachment, migration, proliferation, desired differentiation of specific cell phenotypes throughout the scaffold where new tissue formation is needed (Chaikof et al. 2002; Wei and Ma 2008).

Nanomaterial is a prosperous field in materials science based on the nanotechnology which was first defined by Taniguchi (1974). Nanotechnology, as a tool for fabricating nanomaterials, is the study of the control of matter on an atomic and molecular scale, which has the potential to create many new materials and devices with wide-ranging applications, such as in medicine, electronics, and energy production. The scale of nanomaterials made by nanotechnology is usually smaller than 100 nm meter in at least one dimension (Buzea et al. 2007), though sometimes also smaller than 1 μm, especially in the biological area.

Nanotechnology or the use of nanomaterials may have the answers since only these materials can be a powerful tool to track cell and mimic surface properties (including topography, composite, etc.) of natural tissues or delivery growth factors for tissue regeneracy. Nanomaterials are the materials with complex nanostructures, normally are fabricated by bottom-up or top-down methods. At the nanometer scale, where many biological processes operate, for example, the functional structures on the cell membrane, enzyme reactions, protein dynamics and DNA, all possess some aspect of nanodimensionality (Harrison 2008). With significant advancements in synthetic and modification methodologies, nanomaterials can be modified to desired sizes, shapes, compositions and properties, which can be used as an ideal cell tracking label the cells without toxicity (Solanki et al. 2008). Furthermore, the ECM that the cells interact with also abounds with nanosized features which does not only adjust the behaviors of the cells contacted with, but also influences the other cells and even tissues. These nanosized features, such as the size of fibers, the pores of matrix, and the chemical composition, control the mechanical properties, the cell adhesion, proliferation and even differentiation on the matrix (Harrison 2008).

23.2 Principles

Since tissue regeneracy is a complex and precise process, in which cell behaviors, nanosized structures, chemical components of ECM and cytokines play critical roles. Understanding these critical aspects in the tissue repairing process helps to develop new techniques for fabricating proper materials in the regenerative medicine.

23.2.1 Nanoparticles for Cell Tracking

The importance of tracking cells in regenerative medicine is increasing because of the developing of basic cell therapy science, which is critical for cell delivery optimization and for accurate biodistribution studies (Solanki et al. 2008; Vaccaro et al. 2008).

In general, there are little cells retained in the target site after cell injection, which is found by cell tracking method. Wentworth et al. (2007) labeled skeletal myoblasts, and bone marrow stromal cells with Europium nanoparticles in advance, then the labeled cells were injected in vivo into the rat heart. The results showed that only approximately 15% of the delivered cells were retained shortly after cell injection and the cells kept losing during the following 5 days. Other groups have reported similar cell retention numbers at the therapeutic site after injection, ranging from 5 to 15% (Freyman et al. 2006; Wentworth et al. 2007). By immunohistochemical detection, the author found that the macrophage infiltrate contribute to losses of both cell types (Wentworth et al. 2007).

Nanoparticles, especially iron oxide nanoparticles and quantum dots (QDs), are one of exciting materials for cell labeling, cell tracking and in vivo imaging, because of ease to synthesize in large quantities from various materials using relatively simple methods. The diameter of the nanoparticles can be tuned from several to a few hundred nanometers with controlled size distribution. Among them, QDs are considered as the ideal tool to label cells for tracking the cells, because of broad adsorption spectra, narrow emission spectra, high fluorescent intensity and long fluorescence lifetime (Solanki et al. 2008).

23.2.2 Scaffold for Tissue Regeneracy

As mentioned above, cell lost is a big problem for the cells injection method for tissue repair. It's a really necessary requirement to make cells adhere to the surface of a scaffold which can prevent cell losing, support three-dimensional tissue formation. Furthermore, depending upon the setting, progenitor cells may need to mature into a tissue-specific phenotype, and fully differentiated cells will need to operate with appropriate functional responses (Chaikof et al. 2002).

Actually, the ECM, which is the natural environment for cells growing, is full of nanosized structure. Bone, as an example, is a nanocomposite that consists of a protein network (i.e., collagen, laminin, fibronectin, and vitronectin) and hard inorganic components (hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$) (Webster 2001; Zhang and Webster 2009). Specifically, 70% of the bone matrix is composed of nanocrystalline HA which is typically 20–80 nm long and 2–5 nm thick (Simon 1994). Other protein components in the bone ECM same with the other tissues are also nanometer in dimension. This self-assembled nanostructured ECM in all tissues closely surrounds and affects these cell behaviors, for example, cell adhesion, proliferation and differentiation. Apparently, the design of novel nanomaterials which possess not only excellent mechanical properties but that are also biomimetic in terms of their nanostructure, has become quite popular in order to improve the functions of cells in regenerative medicine (Zhang and Webster 2009).

In 2004, Miller et al. (2004) reported endothelial and vascular smooth muscle cells adhesion and proliferation were enhanced comparing a nanostructured PLGA surfaces with smooth one. Later, a series of PLGA surfaces were fabricated by composing of submicron scale spheres on them. The results revealed that surfaces with 200 nm lateral diameter spherical features exhibited highest fibronectin and collagen type IV adsorption comparing the 100 or 500 nm lateral diameter spherical surface features. Furthermore, the higher fibronectin and collagen IV adsorption, the more endothelial and vascular smooth muscle cell adhesion was found as well (Miller et al. 2007). Since chemistry was similar between all PLGA surfaces investigated, this study provided strong evidence of the influence of nanometer features on optimizing fibronectin interactions and subsequently vascular cell adhesion. Similar results were also found that nanostructured titanium implant surfaces promote bone cell responses leading to accelerated calcium deposition improving integration with surrounding bone compared to conventional titanium surfaces (Ergun et al. 2008; Webster et al. 1999; Yao et al. 2008).

Collagen, the major ECM component of most of these tissues, has been proved as a substrate or scaffold for cell attachment, proliferation, and differentiation (Elsdale and Bard 1972; Strom and Michalopoulos 1982). Moreover, the nanosized collagen fibrillar structure (50–500 nm in diameter) has been demonstrated to enhance cell/matrix interactions (Grinnell and Bennett 1982; Kuntz and Saltzman 1997). For serving as a scaffold for regenerative cells, the nanosized fibers like collagen may be help to improve the cell/scaffold interactions and to be a better environment for cell growing. Several techniques, for example, self-assembly, phase separation, and electrospinning are developed to fabricate porous scaffolds composed with nanosized fibers (Wei and Ma 2008).

For example, electrospun poly(L-lactide-*co*-ε-caprolactone) (PLCL) fibrous scaffolds of varying fiber diameters (ranging from 300 nm to 7 mm) were used as scaffolds for culturing human umbilical vein endothelial cells (HUVECs). A higher cell adhesion and proliferation potential was found cultured with nanosized PLCL scaffolds (Kwon et al. 2005). Ma's group developed a thermally induced phase separation method to fabricate nanofibrous scaffold (Zhang and Ma 1999). With this method, nanofibrous poly(L-lactide) (PLLA) scaffolds with diameters ranging from

50 to 500 nm has been prepared using tetrahydrofuran (THF) as solvent (Zhang and Ma 1999, 2000). Nanofibrous scaffolds of PLLA prepared from this technique have demonstrated to adsorb/absorb cell adhesive proteins (fibronectin and vitronectin) two to four times higher and an almost twofold increased osteoblast attachment in comparison to solid walled PLLA scaffolds (Yang et al. 2004a).

So, an ideal 3D-scaffold for tissue regeneracy should have similarity to native ECM in terms of both chemical composition and physical nanostructure. Nanostructured biomaterials having physical features in the nanometer range, such as nanocrystals, nanofibers, nanosurfaces and nanocomposites, have gained much interest recently in regenerative medicine (Layrolle and Daculsi 2006; Thomas et al. 2006a).

Furthermore, except ECMs, intrinsic regulators (e.g., growth factors and signaling molecules) are another prime factors that have critical roles in the regulation of cell behaviors during the tissue reparation (Kiritsy and Lynch 1993; Solanki et al. 2008). For example, during the cutaneous wound repair process, the growth factors (platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), epidermal growth factor (EGF), and fibroblast growth factor (FGF) et al) play important roles in all of three repair phases (hemostasis and inflammation, granulation tissue formation, and matrix formation and remodeling) (Kiritsy and Lynch 1993). However, direct injection of growth factors solution into a regeneracy site is generally not effective because of its rapid diffusion and short lifetime. To enable growth factors to efficiently exert their biological effects during the tissue regeneracy process, a drug delivery system should be used (Tabata 2003).

23.3 Technological and Biological Opportunities for Therapeutic Devices

23.3.1 Functionalized Nanoparticles for Cell Tracking

Over the past decade, cell tracking is becoming more and more important for optimizing cell delivery or accurate biodistribution studies in regenerative medicine as mentioned above.

Nanoparticles, because of the size-dependent properties and dimensional similarities to biomacromolecules, are suitable as contrast agents (Bruchez-Jr. et al. 1998; Chan and Nie 1998; Chan et al. 2002; Dahan et al. 2003; Dubertret et al. 2002; Ishii et al. 2003; Jaiswal et al. 2002; Lidke et al. 2004) or probes for biomedical imaging (Bulte et al. 2001; Josephson et al. 1999).

Magnetic nanoparticles in magnetic resonance imaging (MRI), QDs and other bioengineered nanoparticles are the commonly used for cell labeling and tracking which provide several unique features and capabilities. Firstly, the size-dependent optical and electronic properties can be tuned continuously by changing the particles size (Alivisatos 1996). Secondly, nanoparticles have big specific surface which can be useful for surface modification in order to target a specific organ or

tissue in human body (Rhyner et al. 2006). These nanoparticles are functionalized by diverse techniques in order to meet the requirements for cell imaging and cell tracking.

23.3.1.1 Magnetic Nanoparticles

Magnetic nanoparticles, especially superparamagnetic iron oxide particles (SPIO), have a variety of applications on molecular and cellular imaging for enhancing magnetic resonance contrast. The popularity of SPIO particle is mainly because of these following several properties: (1) they provide the most change in signal (albeit hypointensity) per unit of metal which can obtain sharp images with different iron concentration; (2) they are composed of biodegradable iron, which is biocompatible and can thus be reused/recycled by cells using normal biochemical pathways for iron metabolism; (3) they can be magnetically manipulated and change their magnetic properties according to size (Bulte and Kraitchman 2004).

Normally, SPIO consist of two components, an iron oxide core and a hydrophilic coating. Typically, the core is magnetite (Fe_3O_4) and/or maghemite ($\gamma\ Fe_2O_3$) which plays important role in the MRI. In some case, SPIO nanoparticles can be functionalized simply by doping some other metal ions during the preparation process. Groman et al. (2007) fabricated one new mixed ferrite colloidal magnetic iron oxides by adding informational atoms (Lanthanide) during formation of the iron oxides core. The new functionalized nanoparticles, not only can be visualized by iron-based MRI, but also can be quantized by neutron activation (Eu, Sm, La, Tb add) and even visualized histologically using time resolved fluorescence (Eu, Tb added).

The SPIO nanoparticles must be functionalized by hydrophilic coating in order to stabilize iron oxide crystals in aqueous colloidal solutions or in vivo, reduce unspecific protein adsorption or cell interactions in vitro or in vivo. Most commonly, surface molecules are biocompatible hydrophilic polymers, for example polysaccharides-dextran (Weissleder and Papisov 1992). A rich dextran density can also enhance circulation time because of the flexible dextran layer forming a "molecular brush" (Papisov et al. 1993) because of the low protein adsorption in plasma. As a fact, long circulation time is critically necessary for better targeting and tracking cells. For this purpose, other biological macromolecules have been investigated for functionalizing iron nanoparticles, e.g. poly(sialic acid), heparin etc., but because of their high cost, efforts have been directed to the design of synthetic hydrophilic macromolecules.

Among these synthetic macromolecules, block-copolymers such as poloxamers and poloxamines has been widely used for enhancing circulation time in vivo because of the effect from poly(ethylene glycol) (PEG) molecules extended in the solution (Moghimi and Hunter 2000). To achieve coupling PEG on iron oxide particles, associated dextran on particles was oxidized and poly-L-lysine (PLL) were attached to the surface by electrostatic force. Finally, methoxy(polyethylene glycol)-O-succinyl succinate was immobilized on PLL covered particles (Weissleder et al. 1995). Then, Butterworth et al. (2001)

developed a new method for the grafting of PEG onto magnetite particles by the use of trimethoxysilane-PEG which is more convenient for controlling the grafting process. The PEG immobilized iron oxide particles produced showed greatly enhanced colloidal stability with respect to uncoated particles.

Targeting specific tissue, organ, or cells precisely are necessary for cellular imaging and tracking. Passive targeting is easier to achieve in application which can make nanoparticles accumulate in a specific tissue or organ (liver, spleen, cancer et al.) by simply controlling the particles size (Thorek et al. 2006). But passive targeting is not a universal method for targeting because of its limitation. In order to achieve active targeting of SPIO against specific tissue, organ or cells, it is necessary to first conjugate targeting agents onto the SPIO surface directly or onto its hydrophilic coating. In this case, reactive moieties (i.e., amines, sulfhydryls, carboxyls, etc.) are needed in order to immobilize targeting moieties (i.e., antibodies, folic acid, galactose etc.). Take dextran coated SPIO particles as an example, these hydroxyl groups on dextran molecules were oxidized by sodium metaperiodate (Weissleder et al. 1995), then further modification can be applied. Josephson et al. (1999) crosslinked dextran with epichlorohydrin, then amination was used to induce amino groups on SPIO particles. Finally, particles were functionalized by tat peptide and fluorochrome for imaging cells (Groman et al. 2007; Josephson et al. 1999; Koch et al. 2003).

In order to improve specific interactions with certain kind of cell, targeting moieties, for example peptides, antibodies, small molecule (folic acid, galactose etc.) are normally immobilized on SPIO particles. HIV tat peptide, which contains a membrane translocating signal, was immobilized on surface for efficiently transporting the iron oxides into cells (Josephson et al. 1999; Koch et al. 2003; Lewin et al. 2000). Monoclonal antibodies (mABs) are the proteins which can only interact with a specific substance, and achieve precisely targeting for one kind of cell. Bulte et al. coupled mouse anti-transferrin receptor mAB OX-26 with magnetic nanoparticles and then magnetically labeled oligodendrocyte progenitors (Bulte et al. 1999) and neural precursor cells (Bulte et al. 2003) by receptor-mediated endocytosis for monitoring cell migration. Schellenberger et al. (2002, 2004) found that Annexin V conjugated nanoparticles could detect apoptotic cells at nanoparticle concentrations as low as 0.1 μg Fe/ml in vitro. Targeting moieties are immobilized with PEG as space, for reducing unspecific interactions and increasing specific interactions. Iron oxide nanoparticle surface was modified by folic acid (FA) with PEG as spacer successfully (Kohler et al. 2004; Sun et al. 2006). Then the cell uptake properties were obviously increased after FA immobilized on particles. The specific interactions was found with FA receptor overexpressed cell line—HeLa, but not with non FA receptor overexpressed cells—MG-63 (Sun et al. 2006).

Moreover, Fe^{2+} released from iron oxide nanoparticles may have potential toxic effects on the cells. In order to prevent Fe^{2+} toxic effects of SPIONs, the gold-coated shell was combined on the surface, which is well know as a stable metal. More importantly, gold has well-defined surface chemistry with thiol or amine moieties. This offers an attractive and convenient route for further functionalization of the SPIONs with biomolecules through thiol- or amine-coupling chemistry (Niemeyer and Ceyhan 2001).

Magnetic nanoparticles labeled cell not only make cell visible in the regenerative medicine, but also may be helpful for guiding cells into usable tissues for transplantation with the help of magnetic fields. Sasaki et al. (2008) fabricated novel magnetic nanoparticles coated with chitosan. When bound to fibroblasts and exposed to an external magnetic field, these magnetic nanoparticles improved cell seeding into the center of a 3D scaffold.

23.3.1.2 Quantum Dots

QDs are crystalline semiconductors typically less than 10 nm in diameter that have been studied for over 20 years. Recently, more and more applications are developed in biomedicine including regenerative medicine field (Bruchez-Jr. et al. 1998; Chan and Nie 1998). In the past decades, several production methods are available, from photolithography to wet chemical synthesis. The QDs produced in colloidal solutions are the most useful for biomedical applications since high-quality nanocrystals can be prepared in large quantities at low costs (Rhyner et al. 2006).

QDs have unique optical and electronic properties comparing with organic dyes and fluorescent proteins because of higher molar extinction coefficients, emission wavelengths size tunable and long term photostability (Cui et al. 2007; Maysinger et al. 2007; Yu et al. 2003). These properties have made QDs a topic of intensive research in tracking cell migration, differentiation and metastasis (Rhyner et al. 2006).

The highest quality QDs are composed of II–VI, IV–VI or III–V semiconductors (Lemon and Crooks 2000; Rogach et al. 1999). The most common QD structure is a CdSe core functionalized with a thin shell of ZnS in order to reduce potential toxicity of core (Rhyner et al. 2006). No acute and obvious CdSe QD toxicity has been detected in studies of cell proliferation and viability in live cells (Derfus et al. 2004; Jaiswal et al. 2002; Parak et al. 2002; Winter et al. 2001) and animal models (Akerman et al. 2002; Larson et al. 2003). However, cytotoxicity was observed when Cd^{2+} was released by oxidization the CdSe in air or UV. This happened when the QD surface coating was not stable enough. But after larger molecules, such as proteins (e.g., streptavidin and bovine serum albumin) are used to functionalize on the surface, slower oxidation is found of the core (Alivisatos et al. 2005). Bioconjugation of QDs with biomolecules, such as arginine-glycine-aspartic acid, did not show any toxic effect on hMSCs as compared with unlabeled human umbilical vein endothelial cells (hMSCs) (Shah et al. 2007).

In general, surface modifications or functionalization must be taken place after the QDs are synthesized in order to transfer to an aqueous phase for medical applications. To accomplish this, the hydrophobic surface ligands can either be exchanged with bifunctional ligands or the entire QD can be coated with an amphiphilic polymer layer. In recent work, Gao and colleagues (2004) encapsulated luminescent QDs with a biocompatible copolymer and linked this amphiphilic polymer to tumor-targeting ligands. Using either subcutaneous injection of QD-tagged cells or systemic injection of multifunctional QD probes, sensitive and multicolor fluorescence

imaging of cells can be achieved under in vivo conditions which may be quite useful for regenerative medicine applications (Rhyner et al. 2006).

Although optical imaging with QDs is highly sensitive, a limitation in depth is a major disadvantage. Other imaging techniques, such as MRI, are more suited for tomography and 3D imaging. By functionalizing on QDs, dual imaging can be achieved. For example, gadolinium, which is visible by MRI, was used to link on the surface of QDs by polymer conjugated lipids (Mulder et al. 2006). These functionalized QDs can be easily detected both by fluorescence imaging and MRI in vitro (Mulder et al. 2006). Furthermore, these Gd-based dual-modality nanoparticle probes are promising for in vivo apoptosis after immobilized Annexin A5 on the surface through a PEG spacer (van-Tilborg et al. 2006). The dual imaging nanoparticle probes can be achieved by linking QDs with Fe_2O_3 or FePt as well (Gu et al. 2004).

Normally, fluorescent QDs require excitation from external illumination sources to fluoresce, which limits their application for imaging living opaque subjects because of the resultant strong autofluorescence background and a paucity of excitation light at non-superficial locations. So et al. (2006) reported self-illuminating quantum dot conjugates designed by mimicking a natural bioluminescence resonance energy transfer (BRET) system, with a mutant of R. reniformis luciferase as the energy donor and quantum dots as the acceptor, and have demonstrated that BRET emission can be imaged in cells and small animals. These self-illuminating QD conjugates can emit long-wavelength (from red to near-infrared) bioluminescent light in living cells and in living animals, even in deep tissues, and can be applied for multiplex in vivo imaging (So et al. 2006).

23.3.1.3 Other Nanoparticles

Except wildly used magnetic nanoparticles and QDs, diverse nanoparticles made by either organic or inorganic are applied as cell tracking or imaging probes.

For the specific application, bioactive inorganic particles, for example hydroxyapatite (HA) particles, were functionalized by fluorescence molecules. Zaheer et al. (2001) synthesized a near-infrared (NIR) fluorescent bisphosphonate derivative that exhibits rapid and specific binding to hydroxyapatite (HA). They demonstrate NIR light–based detection of osteoblastic activity in the living animal, and discuss how this technology can be used to study skeletal development. Fluorescence imaging of osteoblastic activity in living animals has also met with success using an active probe: a tetrasulfonated heptamethine indocyanine conjugated to the hydroxyapatite-binding ligand pamidronate (Rao et al. 2007).

A large group of organic nanoparticles such as liposomes, dendrimers and polymersomes have not only been developed for drug delivery, but can also be applied to in vivo optical imaging. Therien and colleagues (Ghoroghchian et al. 2005) reported the synthesis of NIR-emissive polymersomes (polymer vesicles with a diameter of 50 nm–50 mm) through the cooperative self-assembly of amphiphilic diblock copolymers and conjugated multi(porphyrin)-based NIR fluorophores.

Dendrimers were previously used as carriers for magnetic resonance imaging (MRI) contrast reagents and, recently, McIntyre et al. (2004) designed a polyamidoamine dendrimer-based fluorogenic substrate to image tumorassociated matrix metalloproteinase-7 in vivo. A boronated dendrimer labeled with a vascular endothelial growth factor (VEGF) and an NIR dye Cy5 has been shown to selectively bind upregulated VEGF receptors in mouse breast carcinoma (Backer et al. 2005).

When multiple fluorescent dyes are attached to the same molecule, such as an antibody, the fluorescent intensity can decrease instead of increase owing to dye–dye quenching. However, when a viral capsid is used as the scaffold for labeling, more than 40 Cy5 dyes can be loaded onto a single virus particle via specific chemical coupling and no fluorescence quenching is observed due to the large intermolecular distances (Soto et al. 2006). This approach has resulted in the synthesis of highly fluorescent viral nanoparticles with a defined structure and a size of 30 nm in diameter. The local dye concentration was reported to be as high as 1.8 mM without significant quenching (Wu et al. 2005). Cowpea mosaic virus nanoparticles labeled with Alexa dyes have been used successfully to visualize the vasculature and blood flow and for imaging human fibrosarcoma-mediated tumor angiogenesis in living mouse and chick embryos (Lewis et al. 2006).

23.3.2 Functionalized Nanomaterials for Tissue Regeneracy

Besides multifunctionalized nanoparticles for better understanding tissue regenerative process through cell tracking and cell imaging, artificial tissues could become important for tissue regeneracy. The principles of the design of an ideal 3D scaffold for tissue engineering remain unclear. The scaffolds should mimic the structure, composition and biological functions of native extracellular matrix (ECM) as much as possible. Moreover, most of the scaffold fabrication strategies have not given importance to mimic the nanoscale physical features of the natural ECM. It is well known that cells and proteins interact at the nanoscale (Cao 2008; Thomas et al. 2006a). For instance, researchers have engineered a variety of scaffolds made from nanotubes, nanofibers, and nano composites that can be used to grow lifelike networks of cells from the liver, bladder, kidney, bones and cardiovascular system. These artificial tissues could be developed into new therapies for patients with diseased or damaged organs.

It is still early, but many laboratories are experimenting with a wide variety of nanomaterial scaffolds that can be infused with cells to form artificial tissues, such as bone and liver. It appears possible to repair damaged nerves by injecting them with nanomaterials that form bridge-like lattices. Other nanostructures show promise as foundations for growing three-dimensional networks of blood vessels.

Considerable efforts have been made to develop ideal scaffolds for tissue engineering so far. Various techniques such as solvent casting/particulate leaching (Mikos et al. 1994), gas foaming (Mooney et al. 1996; Nam et al. 2000), and phase separation/emulsification (Nam and Park 1999a, b) have been employed to fabricate

conventional porous polymeric foams. Peptide self-assembling, phase separation, and electrospinning are normally techniques used for fabricating nanostructured scaffold materials.

23.3.2.1 Self-Assembly

Self-assembly is a process in which molecules and supramolecular aggregates organize and arrange themselves into an ordered structure through weak and non-covalent bonds (Murugan and Ramakrishna 2007; Whitesides and Grzybowski 2002). It is a common process in nature, for example, collagen has a triple helix secondary structure, which consists of three polypeptide chains in an extended left handed helix (Ramachandran 1988). Self-assembly could be used to produce natural or synthetic polymers into nanoscale structures including nanofibers (Chiti et al. 2003; Hartgerink et al. 2001; Zhang 2003), especially scaffolds based on peptides and proteins. The biological ECMs made by this technique are able to interact with cells at the molecular level to control the processes of tissue regeneracy effectively.

Several studies report promising results of this strategy. For example, a peptide amphiphile (chemical compound possessing both hydrophilic and hydrophobic properties) nanofiber network could be mineralized with hydroxyapatite to recreate the nanoscale structure of bone (Hartgerink et al. 2001). Certain peptide amphiphiles can be designed in order to get functionalized nanomaterials for specific applications. For example, these amphiphile nanofibers have been designed to mimic the collagen structure-building protein-like structural motifs that incorporate sequences of biological interest (Berndt et al. 1995; Fields et al. 1998; Yu et al. 1998, 1999). These nanofibers have been also applied to promote rapid and selective differentiation of neural progenitor cells into neurons (Silva et al. 2004). Self-assembly was also used successfully to encapsulate chondrocytes within a self-assembled peptide hydrogel scaffold for cartilage repair (Engel et al. 2008; Kisiday et al. 2002). Self-assembly of PAs can be promoted by various factors such as pH change, presence of Ca^{2+} ions, and drying on surface. Hong et al. (2003) developed another kind of peptide containing 16 alternating hydrophobic and hydrophilic amino acids and studies the effect of amino acid sequence and pH on self-assembly into nanofibers (Thomas et al. 2006a).

23.3.2.2 Phase Separation

Phase separation techniques have been used to prepare porous polymer membranes for purification and separation purposes. In last two decades, it is becoming a frequently used and convenient method to prepare porous tissue regenerative scaffolds. A variety of biodegradable polymers have been fabricated into three-dimensional porous scaffolds using phase separation techniques (Gong et al. 2006; Ma et al. 2003; Zhang and Ma 1999). In order to meet the requirement of nanoscaled scaffold for tissue regenerative process, a novel phase separation technique has been developed

to generate nanofibrous structures by manipulating the phase separation process (Chen and Ma 2006; Zhang and Ma 2000). The poly(L-lactic acid) (PLLA) fibrous scaffold contains nanofibers ranging from 50 to 500 nm in diameter (Chen and Ma 2006), which is similar to natural collagen fibers in size (Elsdale and Bard 1972; Hay 1991). Nanofibrous scaffolds of PLLA prepared from this technique have demonstrated to adsorb/absorb cell adhesive proteins (fibronectin and vitronectin) two to four times higher and an almost twofold increased osteoblast attachment in comparison to solid walled PLLA scaffolds (Zhang and Ma 2000). Due to the substantial surface area difference, degradation is much more rapid in such nanofibrous scaffolds, in which the overall mass loss is 51% while mass loss in solid-walled nonfibrous foams is only 6% after 15 months (Chen and Ma 2006).

One limitation of the early nanofibrous materials generated using the phase-separation technique is the lack of interconnected macropores, which are critical for cell seeding and recruiting, mass transfer, vascularization, and tissue organization. To overcome this problem, phase separation techniques are used in combination with other scaffold fabrication techniques such as porogen leaching. The combined technique provides broader control over porous architectures from macro-, micro- to nanoscales (Chen and Ma 2004; Gong et al. 2008; Ma et al. 2005c; Wei and Ma 2006; Zhou et al. 2005). Gong et al. (2008; Zhou et al. 2005) fabricated well connected PLLA scaffolds via porogen leaching with phase separation technique in which gelatin particles was used as porogens. The biological performance of the scaffold was evaluated by in vitro chondrocyte culture and in vivo implantation. In comparison with the control scaffold fabricated with NaC1 particles as porogen under the same conditions, the experimental scaffold had better biological performance because the gelatin molecules were stably entrapped onto the pore surfaces (Gong et al. 2008). Surface modification was also taken place in order to improve the biocompatibility of these PLLA scaffolds. Ma et al. (2005c) immobilized collagen and introduced basic fibroblast growth factor (bFGF) on PLLA scaffold. Chondrocyte culturing on the collagen immobilized PLLA surfaces showed significantly improved cell spreading and growth. Incorporation of fibroblast growth factors in the collagen layer further enhanced the cell growth (Ma et al. 2005c).

23.3.2.3 Electrospinning

Electrospinning, as another method to produce nanoscale fibers, is a simple and cost-effective fabrication process that uses an electric field to control the deposition of polymer fibers onto a target substrate (Engel et al. 2008). The generated fibers can mimic the structural profile of the proteins found in the native ECM. The use of electrospinning process in biomaterials field was first reported by Martin and Cockshott (1977) as early as 1977. Since then, electrospinning process has been continuously investigated for the fabrication of nanofibrous matrices for divers applications (Chiu et al. 2005; Fong et al. 1999; Kim and Reneker 1999; Li et al. 2002; Ma et al. 2005b; Reneker and Chun 1996; Yoshimoto et al. 2003). Various synthetic polymer, PLA (Zeng et al. 2003; Zong et al. 2002), PLGA (Li et al. 2002),

PCL (Gong et al. 2006; Boland et al. 2005), poly(dioxanone) (PDS) (Boland et al. 2005) and synthetic polypeptide (Huang et al. 2000), natural proteins such as collagen (Huang et al. 2001; Matthews et al. 2002, 2003), silk protein (Li et al. 2005), elastin fibrinogen (Wnek et al. 2003) etc., are used for fabricating biodegradable scaffold. Electrospun nanofibers have been shown to support cell attachment and proliferation of a variety of cells as they have large surface area and well-interconnectivity of inter-fiber spaces, in addition to the nano sized diameters mimicking the physical nanoscaled dimensions of native ECM (Thomas et al. 2006a).

Biomolecules such as growth factors, drugs, and genes can be directly mixed into the polymer solution and electrospun to prepare functionalized polymer nanofibers. These functionalized bioactive nanofibers have potential applications in both tissue regeneracy and drug delivery systems. Co-spinning of growth factors for cells in future may enable to fabricate scaffolds with controlled release of cellular nutrients. Luu et al. (2003) and Liang et al. (Ye and Huang 2005) have encapsulated plasmid DNA in PLA-PEG and co-electrospun with PLGA in DMF and electrospun the mixture into nanofibers. Release of plasmid DNA from the scaffolds was studied for 20 days and found that the release of DNA sustained over 20 day period with a maximum release occurring at 2 h. Verreck et al. (2003) prepared polyurethane nanofibers containing model drugs itraconazole and ketanserin to study the pattern of drug release. Co-spinning of growth factors for cells in future may enable to fabricate scaffolds with controlled release of cellular nutrients (Thomas et al. 2006a).

Bioactive nanoscale fillers, e.g. hydroxyapatite (HA), tricalcium phosphate (TCP) et al., are incorporated into polymer solution to electrospun nanocomposite nanofibers for better interactions with cells. Thomas et al. (2006b) examined the physical property changes after nanoHA incorporated into PCL nanofibers. They demonstrated that it is possible to tailor subtle mechanical properties in a nanofibrous matrix by incorporating nanofillers of desired amount. Higher percentage loadings of nanoHA resulted in poor dispersion of the nanoHA powder as particle size of nanoHA used was ~100 nm. If the particle size of HA is small enough (~20–40 nm), PCL/nanoHA composite with more than 20-wt% produces fibers with well dispersed nanoHA (Thomas et al. 2006b). It has been reported that chondrocyte adhesion and proliferation on polymer/nanoHA composite materials are better than the pure polymer (Hong et al. 2005). MSCs seeded onto nanocomposite scaffolds exhibited well cell spreading and growth on PCL/nanoHA nanocomposites, revealing favorable cell-matrix interactions (Thomas et al. 2006a).

As mentioned earlier, electrospinning of collagen into nanofibers have opened the door to make nanofibrous matrices mimicking nano structures of bone for bone tissue engineering. However an ideal scaffold for bone tissue engineering should mimic not only the nanofibrous physical structure but also the chemical composition. Electrospun nanofibrous nanobiocomposite scaffolds based on Type I collagen and nanoHA have been prepared as biologically inspired scaffolds mimicking the chemical and morphological features of natural ECM (Thomas et al. 2007).

Very recently, Badami et al. (2006) have electrospun PLA as well as PEG-PLA di-block copolymers of PEG-PLA into fibers with diameters ranging from 140 nm to 2.1 mm and cultured MC3T3-E1 mouse calvaria-derived osteoprogenitor cells on the

scaffolds up to 14 days. The results of study focussed on the effect of fiber diameter on spreading, proliferation and differentiation of osteoblastic cells on fibrous scaffolds with and without osteogenic factors. The authors concluded that in the absence of osteogenic factors such as β-glycerophosphate and L-ascorbat-2-phosphate, cell growth (cell density) was lower on polymer fibers than smooth polymer surfaces, while in the presence of osteogenic factors cell density on fibers was equal or greater than that on smooth surfaces (Thomas et al. 2006a).

Venugopal et al. (2005) have coated collagen over electrospun PCL by soaking the PCL matrix in collagen solution (10 mg/mL) and cultured human coronary artery smooth muscle cells. It was observed that SMCs migrated towards inside the nanofibrous matrices and formed smooth muscle tissue in 72 h. According to the authors, PCL scaffold supporting the cell growth needs collagen support for migration of cells inside the nanofibrous matrices. In another study He et al. (2005) showed that collagen coated PLLA-CL nanofibers exhibited enhanced cell attachment, spreading and viability of human coronary artery endothelial cells. It was found that coating of collagen on PCL scaffold definitely favored cell proliferation.

The co-use of these adhesion proteins and biodegradable synthetic polymers enables the construction of cell-adhesive scaffolds for vitally functioning engineered tissues (Almany and Seliktar 2005; Chen et al. 2000; Kwon et al. 2001; Kwon and Matsuda 2005; Zhang et al. 2005a). Co-electrospinning is a feasible approach to provide a compromise solution for overcoming the shortcomings of synthetic and natural polymers that is producing new porous nanofibrous biomaterials with good biocompatibility and improved mechanical, physical and chemical properties and biological performance.

Stitzel et al. (2006) have recently fabricated a vascular graft scaffold from electrospun polymer blends of Type I collagen (45 wt%), elastin (15 wt%) and PLGA (45 wt%). They found that by controlling the compositional ratio of collagen, elastin, and PLGA have resulted in improved electrospun fiber characteristics and physical strength of the vascular graft.

Core-shell types of multi component nanofibers by co-axial electrospinning are of another interesting mixed polymer system in tissue engineering for bioactive scaffolds. Functionalization of fibers without affect the core is desirable in tissue engineering and in controlled drug delivery for preserving an unstable biological agent from an aggressive environment and delivering a biomolecular drug in a sustained way. Co-axial electrospinning is a method for incorporation of water-soluble macromolecules as the core of nanofibers during electrospinning. The production of core-shell nanofibers from co axial electrospinning was first demonstrated by Sun et al. (2003). Zhang et al. (2004) fabricated bi-component nanofibers of PCL and gelatin in the form of a core-shell structure by coaxial electrospinning. A quantitative analysis of the effect of gelatin concentration on the diameters of core and shell of nanofibers was carried out that when the concentration of gelatin was below 12.5 w/v% the diameter of core and shell were, respectively, less than 200 and 400 nm. Zhang et al. (2005b) have fabricated collagen-PCL nanofibrous scaffold (collagen-r-PCL) by coaxial electrospinning and compared the surface biocompatibility

with electrospun neat PCL, electrospun neat collagen scaffold and collagen coated PCL scaffolds by culturing human dermal fibroblasts. As compared to neat PCL scaffold and collagen coated PCL, scaffold human dermal fibroblasts cell density on collagen-r-PCL linearly increased over period. However, cell proliferations data of collagen-r-PCL are not significantly differ from those of electrospun neat collagen. In another study Jiang et al. (2005) fabricated biodegradable core-shell nanofibers with PCL as shell and protein containing PEG as core for controlled release of incorporated proteins such as lysozyme and BSA.

Recently, a group based in Singapore developed an alternative approach to wound healing, which they termed autologous layered dermal reconstitution (ALDR) (Chong et al. 2007). This technique relies upon novel TE scaffolds which consist of electrospun fibers made of PCL and gelatin, between 300 and 600 nm in diameter, with a total thickness of only 28 μm. The scaffolds were seeded with human dermal fibroblasts, which remained viable in the scaffold for all time points tested (up to 2 weeks) and doubled in population approximately every 3 days. Although no in vivo results are currently available, ALDR using electrospun scaffolds should offer a distinct advantage over traditional techniques. Namely, ALDR will allow for a rapid, layer-by-layer buildup of tissue in deep wounds, with dermal fibroblasts distributed throughout. This can occur because the electrospinning process takes place on top of a commercially available polyurethane wound dressing. As little as 48–72 h after implantation, the wound dressing can be removed, and another scaffold/wound dressing construct placed in the wound site. This is repeated until the wound area is fully repaired. Since each scaffold will be individually seeded with dermal fibroblasts prior to implantation, this layer by layer technique eliminates the long in vitro culture times otherwise needed for cellular infiltration and growth within larger, single-layer scaffolds. The end result is a continuous layer of tissue, wherein the use of a porous, nanostructured scaffolds allows for rapid cellular proliferation and integration between layers (Khang et al. 2010).

23.3.2.4 Nanocomposite Scaffold

For successful cell-based therapy, one major obstacle is the low cell engraftment and viability after transplantation (Li et al. 2007a). Cellular microenvironment especially extracellular matrix (ECM) is one of the essential factors for cell local activity, such as adhesion, differentiation and proliferation. Many functional molecules contained in ECM and their interaction with cells are crucial to regulate cell survival, renewal and maintaining of cell capacity. Tissue engineered matrices for the homing and support of transplanted cells is of great interests recent years (Bonadio et al. 1999; Wang et al. 2009). Hence a wide range of natural and synthetic extracellular matrices with good biocompatibility are selected for tissue engineering purposes.

Nanocomposite scaffold are made of regenerative scaffold with certain nanostructure system, for example, bioactive molecules and particles. Nanocomposites can be reinforced polymers or ceramics with low quantities of nanometric-sized

particles (silicate, carbon nanotubes (CNT)) which give them improved properties. The properties of nano-composite materials depend not only on the properties of their individual parents but also on their morphology and interfacial characteristics. This rapidly expanding field is generating many exciting new materials with novel properties. Nanocomposites have attracted a great deal of attention in biomedical applications also. Many natural tissues such as bone possess a composite micro/nano structure. These complex composite structures play roles for the physical and biological properties of the tissues. To mimic the natural tissue structure, biomedical polymers, bioceramics and other organic/inorganic materials are to be combined for superior properties. Composite materials often show an excellent balance between strength and toughness and usually improved characteristics compared to their separate components. Recently, Kothapalli et al. have shown that by incorporation of 50 wt% nanoHA into PLA scaffold, the yield strength increased 150% and compression modulus almost doubled in comparison to pure PLA. Addition of nanoHA can improve osteoconductivity to the polymer scaffolds (Kothapalli et al. 2005).

One of the most interested nanocomposite biomaterials for bone regeneracy is comprised of biodegradable polymers with nanoHA or other calcium phosphate bioceramic composition. Experiments prove that nanometer features on biomaterial surfaces can be used to guide cell behavior along a desired biological response (Liu and Webster 2007; Webster et al. 1999). In bone-regeneracy applications, promising results have been obtained with the nanophase materials ceramics and metals, with which increased osteoblast adhesion, proliferation and calcium deposition have been observed compared with conventional materials (i.e. with micrometer-scaled grains) (Webster and Ejiofor 2004).

Synthetic extracellular matrix could be easily prepared and owns good biocompatibility for cell tissue engineering purposes. However, synthetic materials have been inferior to natural extracellular matrix scaffolds that allow regeneration to occur. Also synthetic materials could be rigid, elicit a mechanical or frictional irritation, limit cell mobility and vascularization; whereas collagen scaffold offers good cytocompatibility and known structural physical and chemical properties. In addition it offers several advantages for stem cell homing and migration. First, collagen gels as scaffold could provide a three-dimensional microenvironment in which cells can grow which mimic the cells' natural in vivo environment. Second, as a natural material, it minimizes the foreign body inflammatory response to the surrounding tissue and favors cell engraftment. Third, the degradation rate of collagen could be easily controlled with noncytotoxic cross-linking agents such as carbodiimide (van Wachem et al. 2001). Furthermore, collagen is also chemotactic to home mesenchymal stem cells (Lewus and Nauman 2005), fibroblast (Gentleman et al. 2004) and other cell types (Gentleman et al. 2006) for tissue regeneration.

Biologically inspired nanobiocomposites of collagen and nanoHA for bone substitute have a long history in biomedical field (Clarke et al. 1993; Itoh et al. 2001; Rovira et al. 1993; TenHuisen et al. 1995). There is possibility of enhancing the functionalities of collagen by incorporating other bone materials such as HA, bone morphogenic proteins (BMP) etc. A combination of collagen and nanoHA materials

is bioactive, osteoconductive and osteoinductive and seems to be a natural choice for bone grafting, i.e., it mimics the bone components. The unique characteristics of this biocomposite is the spatial orientation between HA and collagen macromolecules, which seems to be the source of the mechanical strength of the composite. Conventionally, collagen/HA nanocomposites can be made by blending or mixing the collagen and HA or by biomimetic methods (Du et al. 1999; Itoh et al. 2004; Liao et al. 2004; Tampieri et al. 2003; Yang et al. 2004b). However most of the collagen/HA composites are conventionally processed by anchoring microHA particles into the matrix of collagen, which makes it quite difficult to obtain a uniform to a homogeneous composite graft. Further, large size crystalline microHA, which is in contrast to natural bone apatite, may take a longer time to remodel into bone tissue up on implantation. In addition some of the composites exhibit very poor mechanical properties, probably due to the lack of strong interfacial bonding between constituents. There is a chance for improving osteointegration by reducing the grain size HA particles by activating the nucleation of ultra fine apatite growth into the matrix. This may lead to enhance mechanical properties and osteointegration with improved biological and biochemical affinity to the host bone (Thomas et al. 2006a).

Nanoparticle within regenerative medicine has been addressed mainly towards the development of entrapment and delivery systems for genetic material, biomolecules, such as growth and differentiation factors, and bone morphogenetic proteins and also as reinforcing- or bioactivity-enhancement phase for polymeric matrices in 3D scaffolds for tissue regeneracy (Engel et al. 2008).

Controlled delivery of biomolecules is crucial in the support and enhancement of tissue growth in tissue regeneracy applications. Nanotechnology approaches in delivery systems can enhance the success of specific therapeutic agents, such as growth factors and DNA among others, which are of paramount importance for tissue regeneracy (Reddy et al. 2006). Carriers in the nanoscale enable the intracellular delivery of molecules and the possibility of reaching targets that are inaccessible normally, such as the blood–brain barrier, tight junctions and capillaries, whereas the control over biomolecule dosage and delivery period are increased. The ultimate challenge is to develop artificial nanocarriers that can target cells with efficiency and specificity similar to that of viruses (Mastrobattista et al. 2006).

Examples of nanoparticles for delivery systems include currently microspheres, microcapsules, liposomes, micelles and also dendrimers. The different types of nanoparticles have been developed as solid, hollow or porous. The most common development methods are molecular self-assembly, nanomanipulation, bioaggregation and photochemical patterning (Allemann et al. 1993; Cade et al. 2004).

Biodegradable polymers are the most commonly used materials in drug delivery. Polylactic acid (PLA), polyglycolic acid (PGA), polyethylene glycol (PEG) and its copolymers have been used widely in combination with hydrogels to attain nanocarriers that exhibit different release properties. Particularly important for the development of nanoparticles for delivery purposes are ‘smart’ or ‘stimuli-responsive’ polymers that can undergo conformational changes, such as swelling or shrinkage, on variations in temperature, pH and magnetic field (Engel et al. 2008).

In sophisticated tissue-engineering strategies, the biodegradable scaffold is preferred to serve as both a 3D substrate and a growth factor delivery vehicle to promote cellular activity and enhance tissue neogenesis (Jain 2008). A novel approach has been described for fabrication of tissue-engineering scaffolds capable of controlled growth factor delivery whereby growth factor containing microspheres are incorporated into 3D scaffolds with good mechanical properties, well-interconnected macroporous and nanofibrous structures (Wei et al. 2006).

Incorporation of microspheres into scaffolds significantly reduced the initial burst release. Sustained release from several days to months was achieved through different microspheres in scaffolds. Released platelet derived growth factor (PDGF) was demonstrated to possess biological activity as evidenced by stimulation of human gingival fibroblast DNA synthesis in vitro. The successful generation of 3D nanofibrous scaffold incorporating controlled-release factors indicates significant potential for more complex tissue regeneracy (Jain 2008). Growth factors are able to be incorporated on regenerative scaffold by other techniques, e.g. layer-by-layer self assembly. Collagen scaffolds functionalized with acid fibroblast growth factor (aFGF) or basic fibroblast growth factor (bFGF) via assembly with heparin/PEI or chondroitin sulfate (Ma et al. 2007; Mao et al. 2005). The results prove that both aFGF and bFGF can be successfully deposited onto the scaffold. The FGFs in the multilayers obviously enhances fibroblast proliferation and viability (Ma et al. 2007; Mao et al. 2005). We show here that the bioactive aFGF has been successfully deposited onto the TCPS sheet surface in the presence of heparin via a layer-by-layer manner. The aFGF built in the multilayers obviously enhances fibroblast proliferation and viability.

The aim of stem cell tissue engineering is generating new tissue to repair damaged tissues or organs by combining biofunctional materials with stem cells which could be administrated by direct transplantation or stimulating stem cell homing to the injured site. SDF-1α is one of the pivotal signals which can guide mature and immature stem/progenitor directional migration towards the high SDF-1α gradient and protect the stem cell from apoptosis (Jaleel et al. 2004). The transient up-regulation of SDF-1α level in damaged organ after injury were identified and it could stimulate stem cell mobilization from bone marrow to the injured area (Klopsch et al. 2009; Ma et al. 2005a). However, SDF-1α could be inactivated and cleaved in a very short half-life (<15 min) by both matrix metalloproteinase-2 (MMP-2) and CD26/dipeptidyl peptidase IV, which are two abundant proteases under inflammatory conditions (De La Luz Sierra et al. 2004; McQuibban et al. 2001; Peterson et al. 2000). Therefore, it is important to sustained release active SDF-1α with a controlled manner. Wang et al. (2010) immobilized the SDF-1α /PEI complexes into a collagen scaffold forming a collagen-based gene activated substrate to provide the localized release of homing signals SDF-1α protein which promoted stem cell homing and recruitment. They optimized transfection efficiency with a high specificity based on the collagen amount, N/P ratio and DNA dosage. They demonstrated SDF-1α secreted by the transfected cells enhanced stem cell recruitment in flow chamber. In vivo, the SDF-1α gene activated matrix could recruit $CD117^{+}$ stem cells after hind limb implantation. More importantly no evidence of inflammation associated with gene activated substrate implantation was

detected, suggesting the gene activated substrate implantation would not increase the inflammation-induced matrix metalloproteinases and other proteinases, consequently the localized released SDF-1α from transfected cells on the gene activated substrate could home the stem cells to the substrate for stem cell adhesion and proliferation on the surface of the matrices.

For the skin tissue engineering, Chung et al. (2006) explored the use of poly (ε-caprolactone) (PCL) grafted with nanostructured chitosan (CS) as a regenerative scaffold for the growth of human dermal fibroblasts. Resultant nano-CS/PCL surfaces exhibited significantly higher surface roughness values as compared to smooth CS/PCL surfaces: 106.0 nm compared to 3.6 nm, respectively. Furthermore, these nano-CS/PCL constructs exhibited significantly ($p < 0.001$) higher rates of fibroblast proliferation and viability as compared to smooth CS/PCL surfaces or nano-rough PCL surfaces. As such, the technique of solvent spin-etching for polymers may represent an inexpensive means to prepare nanoscale TE scaffolds as improved artificial skin grafts (Khang et al. 2010).

23.4 Applications for Therapeutic Devices

After several decades development, dextran and other polymer-coated SPIONs are currently used in a number of biomedical applications; for example, Endorem® (Geurbet, France) is a commercially available contrast agent based on SPIONs surface coated with dextran (Corot et al. 2006). It is a suitable contrast agent for labeling human MSCs (hMSCs) and human ESCs (hESCs) as it does not need a transfection agent (which may damage the stem cells) to facilitate its cellular uptake. Feridex ® and Sinerem® are other commercially available dextran-coated SPIONs that are combined with commercially available transfection agents, such as Fungene™, Superfect™ or Lipofectamine (Bulte and Kraitchman 2004; Corot et al. 2006). The use of transfection agents at higher concentrations may increase toxicity and, at lower concentrations, may not lead to sufficient cellular uptake (Bulte and Kraitchman 2004). Thus, the amount of transfection agent needed to enhance internalization is optimized carefully before combining it with SPIONs. The amount also depends on the stem cell type to be labeled. Li et al. optimized the conjugation of PEI onto the surface of the magnetic nanoparticles. The magnetic nanoparticles could enhance the transfection efficiency and effectively deliver genes to the left side of the mouse thorax underexternal magnetic guidance (Li et al. 2007b, 2008).

With the further development and investigation, more and more products will be commercially available.

23.5 Barriers to Practice and Prospects

Although research on nanoparticles for non-invasive detecting is developing continually, there are still a lot of barriers which should be overcome.

Every technique and or application has it limitations, and the use of iron oxides or quantum dots for molecular and cellular imaging is no exception. (1) Resolution of MRI and fluorescence imaging is not good enough. For better understanding of cell behaviors, high resolution is required to investigate one single cell's migration, proliferation, and differentiation. (2) Better targeting and lower dose for imaging. Highly specific targeting is necessary for labeling interested cells only in order to reduce dose and get higher resolution. (3) For cellular imaging, as labeling is not permanent and self-replicable like reporter genes, with dilution of label upon cell division, iron oxide detection may rapidly become impossible, both in vitro (Bulte et al. 2001; Schaffer et al. 1993) and in vivo. (4) Finally, careful iron oxide titration and cellular differentiation studies need to be performed, as labeling may lead to inhibition of differentiation into certain cell types, without affecting cell viability or proliferation (Kostura et al. 2004). Standardized protocol for phenotypic and genotypic characterization of mesenchymal stem cells could be a reference for the efficacy and safety considerations of nanoparticles in clinical applications to ensure the cells could be sustainable propagation without alterations in their genetic traits and functional capabilities (Furlani et al. 2009).

For the scaffold for tissue regeneracy, they have not been used extensively but major contributions are expected in two areas. The first is growth of complex tissue, where microfluidic structures ensure a steady blood supply, thereby circumventing the well-known problem of providing larger tissue structures with a continuous flow of oxygen as well as nutrition and removal of waste products. The second, and probably more important function of microfluidics, combined with micro/nanotechnology, lies in the development of in vitro physiological systems for studying fundamental biological phenomena (Jain 2008).

23.6 Conclusions and Future Challenges

Nanomaterials are considered as a new class of materials possessing superior properties over its microscale counterparts. Nanostructured biomaterials having physical nanofeatures such as nanocrystals, nanofibers nanosurfaces, nanocomposites, etc. have gained much interest in regenerative medicine.

The coregistration of in vivo fluorescence imaging with anatomical imaging modalities such as MRI helps traverse the shortcomings of fluorescence imaging, such as limited tissue penetration of photons and low three-dimensional spatial resolution, and provides complementary information. The development of multifunctional probes is attracting increasing attention and several studies have already appeared – from iron-oxide- and dendrimer-based dual MRI–fluorescence imaging contrast agents.

Effective and innovative imaging approaches are in great demand as new proteins and genes, particularly within the field of oncology, are being discovered at an ever-increasing pace. This provides a constantly multiplying library of molecules and

pathways to be studied for prevention, diagnosis, and treatment of diseases. The full potential of new discoveries is however limited by the void between the advances in bioscience and the means to accurately, effectively and – critically – non-invasively image the molecular interactions in biological systems. Many challenges clearly remain in the pursuit of ideal SPIO probes for molecular imaging; increased target affinity, less complex conjugation schemes, reduction of cost, a means for MRS to avoid sequestration in lysosomes and more effective activatable probes. With persistent advances, this system continues to demonstrate its potential as a means to probe deeper into our biological universe.

Nanostructured scaffolds are interested in regenerative medicine, mainly because of their resemblance of nanomorphology and physical nanofeatures to natural extra cellular matrices. The nanoscaled features such as surface roughness and topography of nanocrystalline bioceramics and nanofibrous scaffolds promote the cell behavior such as adhesion, proliferation and migration and differentiated functions. Polymeric nanofiber based nonwoven matrix is among the most promising nanostructured biomaterials for native ECM analogs. Electrospinning is a versatile technique to fabricate nanofibrous matrices of polymers for tissue engineering scaffold applications. One of the particular advantages of electrospinning in regenerative medicine is the ability to co-spin various components such as cell adhesive proteins and other cell-growth factors along with biodegradable synthetic or biopolymers. An ideal 3D-scaffold for tissue engineering should have similarity to native ECM in terms of both chemistry and physical nanostructure. Electrostatic co-spinning of nanocomposite fibers of polymers with nanoHA to fabricate hybrid scaffolds of improved mechanical properties and cellular behaviors has been established in our group. The unique characteristics of collagen/nanoHA composite system in native bone is the special orientation between HA and collagen molecules. Therefore, future efforts in nanofibrous collagen/nanoHA composite are required mimicking exactly the complex nano structured architecture of collagen matrix with the c-axis orientation of nanoHA particles (Thomas et al. 2006a).

Regenerative medicine aspects that focus on TE have evolved into two main strategies. The first strategy consists of an elegant approach in which stem cells harvested from the patient are expanded and seeded on 3D scaffolds within a bioreactor. The resulting hybrid construct is then implanted into the patient (together with growth factors) as a tissue matrix. However, the need to harvest and expand stem cells poses great efficacy and efficiency problems that define the success of the entire process. The second strategy relies on the development of intelligent materials that would be able to send signals to the stem cells already present in the diseased or damaged tissue niches that would then trigger the regeneracy process. Nanotechnology is a powerful tool for creating these ‘smart’ materials. This approach is challenging and is still far from being achieved. Among other advantages, it would raise the possibility to have such cell-free materials ready ‘off the shelf’ and to be able to use them as and when required (Engel et al. 2008).

References

Akerman ME, Chan WC, Laakkonen P et al (2002) Nanocrystal targeting in vivo. Proc Natl Acad Sci 99:12617–12621

Alivisatos AP (1996) Semiconductor clusters, nanocrystals, and quantum dots. Science 271:933–937

Alivisatos AP, Gu W, Larabell C (2005) Quantum dots as cellular probes. Annu Rev Biomed Eng 7:55–76

Allemann E, Gurny R, Doelker E (1993) Drug-loaded nanoparticles: preparation methods and drug targeting issues. Eur J Pharm Biopharm 39:173–191

Almany L, Seliktar D (2005) Biosynthetic hydrogel scaffolds made from fibrinogen and polyethylene glycol for 3D cell cultures. Biomaterials 26:2467–2477

Backer MV, Gaynutdinov TI, Patel V et al (2005) Vascular endothelial growth factor selectively targets boronated dendrimers to tumor vasculature. Mol Cancer Ther 4:1423–1429

Badami AS, Kreke MR, Thompson MS et al (2006) Effect of fiber diameter on spreading, proliferation, and differentiation of osteoblastic cells on electrospun poly(lactic acid) substrates. Biomaterials 27:596–606

Berndt P, Fields GB, Tirrell M (1995) Synthetic lipidation of peptides and amino acids: monolayer structure and properties. J Am Chem Soc 117:9515–9522

Boland ED, Coleman BD, Barnes CP et al (2005) Electrospinning polydioxanone for biomedical applications. Acta Biomater 1:115–123

Bonadio J, Smiley E, Patil P et al (1999) Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nat Med 5:753–759

Bruchez-Jr M, Moronne M, Gin P et al (1998) Semiconductor nanocrystals as fluorescent biological labels. Science 281:2013–2016

Bulte JWM, Kraitchman DL (2004) Iron oxide MR contrast agents for molecular and cellular imaging. NMR Biomed 17:484–499

Bulte JWM, Zhang S-C, Pv G et al (1999) Neurotransplantation of magnetically labeled oligodendrocyte progenitors: magnetic resonance tracking of cell migration and myelination. Proc Natl Acad Sci 96:15256–15261

Bulte JW, Douglas T, Witwer B (2001) Magnetodendrimers allow endosomal magnetic labeling and in vivo tracking of stem cells. Nat Biotechnol 19:1141–1147

Bulte JWM, Ben-Hur T, Miller BR et al (2003) MR microscopy of magnetically labeled neurospheres transplanted into the Lewis EAE rat brain. Magn Reson Med 50:201–205

Butterworth MD, Illum L, Davis SS (2001) Preparation of ultrafine silica- and PEG-coated magnetite particles. Colloid Surf A 179:93–102

Buzea C, Blandino IIP, Robbie K (2007) Nanomaterials and nanoparticles: sources and toxicity. Biointerphases 2:17–71

Cade D, Ramus E, Rinaudo M et al (2004) Tailoring of bioresorbable polymers for elaboration of sugar-functionalized nanoparticles. Biomacromolecules 5:922–927

Cao YC (2008) Nanomaterials for biomedical applications. Nanomedicine 3:467–469

Chaikof EL, Matthew H, Kohn J et al (2002) Biomaterials and scaffolds in reparative medicine. Ann NY Acad Sci 961:96–105

Chan WCW, Nie S (1998) Quantum dot bioconjugates for ultrasensitive nonisotopic detection. Science 281:2016–2018

Chan WCW, Maxwell DJ, Gao X et al (2002) Luminescent quantum dots for multiplexed biological detection and imaging. Curr Opin Biotechnol 13:40–46

Chen VJ, Ma PX (2004) Nano-fibrous poly(L-lactic acid) scaffolds with interconnected spherical macropores. Biomaterials 25:2065–2073

Chen VJ, Ma PX (2006) The effect of surface area on the degradation rate of nano-fibrous poly(l-lactic acid) foams. Biomaterials 27:3708–3715

Chen G, Ushida T, Tateishi T (2000) Hybrid biomaterials for tissue engineering: a preparative method for PLA or PLGA-collagen hybrid sponges. Adv Mater 12:455–457

Chiti F, Stefani M, Taddei N et al (2003) Rationalization of the effects of mutations on peptide and protein aggregation rates. Nature 424:805–808
Chiu JB, Luu YK, Fang D et al (2005) Electrospun nanofibrous scaffolds for biomedical applications. J Biomed Nanotechnol 1:115–132
Chong EJ, Phan TT, Lim IJ et al (2007) Evaluation of electrospun PCL/gelatin nanofibrous scaffold for wound healing and layered dermal reconstitution. Acta Biomater 3:321–330
Chung T-W, Wang Y-Z, Huang Y-Y et al (2006) Poly (ε-caprolactone) grafted with nano-structured chitosan enhances growth of human dermal fibroblasts. Artif Organs 30:35–41
Clarke KI, Graves SE, Wong ATC et al (1993) Investigation into the formation and mechanical properties of a bioactive material based on collagen and calcium phosphate. J Mater Sci Mater Med 4:107–110
Corot C, Robert P, Idée J-M et al (2006) Recent advances in iron oxide nanocrystal technology for medical imaging. Adv Drug Deliv Rev 58:1471–1504
Cui B, Wu C, Chen L et al (2007) One at a time, live tracking of NGF axonal transport using quantum dots. Proc Natl Acad Sci 104:13666–13671
Daar AS, Greenwood HL (2007) A proposed definition of regenerative medicine. J Tissue Eng Regen Med 1:179–184
Dahan M, Lévi S, Luccardini C et al (2003) Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking. Science 302:442–445
De La Luz SM, Yang F, Narazaki M et al (2004) Differential processing of stromal-derived factor-1alpha and stromal-derived factor-1beta explains functional diversity. Blood 103:2452–2459
Derfus AM, Chan WCW, Bhatia SN (2004) Probing the cytotoxicity of semiconductor quantum dots. Nano Lett 4:11–18
Du C, Cui FZ, Zhu XD et al (1999) Three-dimensional nano-HAp/collagen matrix loading with osteogenic cells in organ culture. J Biomed Mater Res A 44:407–415
Dubertret B, Skourides P, Norris DJ et al (2002) In vivo imaging of quantum dots encapsulated in phospholipid micelles. Science 298:1759–1762
Elsdale T, Bard J (1972) Collagen substrata for studies on cell behavior. J Cell Biol 54:626–637
Engel E, Michiardi A, Navarro M et al (2008) Nanotechnology in regenerative medicine: the materials side. Trends Biotechnol 26:39–47
Ergun C, Liu HN, Webster TJ et al (2008) Increased osteoblast adhesion on nanoparticulate calcium phosphates with higher ca/p ratios. J Biomed Mater Res A 85A:236–241
Fields GB, Lauer JL, Dori Y et al (1998) Proteinlike molecular architecture: biomaterial applications for inducing cellular receptor binding and signal transduction. Peptide Sci 47:143–151
Fong H, Chun I, Reneker DH (1999) Beaded nanofibers formed during electrospinning. Polymer 40:4585–4592
Freyman T, Polin G, Osman H et al (2006) A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction. Eur Heart J 27:1114–1122
Furlani D, Li W, Pittermann E et al (2009) A transformed cell population derived from cultured mesenchymal stem cells has no functional effect after transplantation into the injured heart. Cell Transplant 18:319–331
Gao X, Cui Y, Levenson RM et al (2004) In vivo cancer targeting and imaging with semiconductor quantum dots. Nat Biotechnol 22:969–976
Gentleman E, Nauman EA, Dee KC et al (2004) Short collagen fibers provide control of contraction and permeability in fibroblast-seeded collagen gels. Tissue Eng 10:421–427
Gentleman E, Nauman EA, Livesay GA et al (2006) Collagen composite biomaterials resist contraction while allowing development of adipocytic soft tissue in vitro. Tissue Eng 12:1639–1649
Ghoroghchian PP, Frail PR, Susumu K et al (2005) Near-infrared-emissive polymersomes: self-assembled soft matter for in vivo optical imaging. Proc Natl Acad Sci 102:2922–2927
Gong Y, Ma Z, Gao C et al (2006) Specially elaborated thermally induced phase separation to fabricate poly(L-lactic acid) scaffolds with ultra large pores and good interconnectivity. J Appl Polym Sci 101:3336–3342

Gong Y, Ma Z, Zhou Q et al (2008) Poly(lactic acid) scaffold fabricated by gelatin particle leaching has good biocompatibility for chondrogenesis. J Biomater Sci Polym Ed 19:207–221
Grinnell F, Bennett MH (1982) Ultrastructural studies of cell–collagen interactions. Meth Enzymol 82A:535–544
Groman EV, Bouchard JC, Reinhardt CP et al (2007) Ultrasmall mixed ferrite colloids as multidimensional magnetic resonance imaging, cell labeling, and cell sorting agents. Bioconjugate Chem 18:1763–1771
Gu H, Zheng R, Zhang X et al (2004) Facile one-pot synthesis of bifunctional heterodimers of nanoparticles: a conjugate of quantum dot and magnetic nanoparticles. J Am Chem Soc 126:5664–5665
Harrison BS (2008) Applications of nanotechnology. In: Atala A, Lanza R, Nerem R, Thomson JA (eds) Principles of regenerative medicine. Academic Press, New York
Hartgerink JD, Beniash E, Stupp SI (2001) Self-assembly and mineralization of peptide-amphiphile nanofibers. Science 294:1684–1688
Hay ED (1991) Cell biology of extra cellular matrix. Plenum Press, New York
He W, Ma Z, Yong T et al (2005) Fabrication of collagen-coated biodegradable polymer nanofiber mesh and its potential for endothelial cells growth. Biomaterials 26:7606–7615
Hong Y, Legge RL, Zhang S et al (2003) Effect of amino acid sequence and pH on nanofiber formation of self-assembling peptides EAK16-II and EAK16-IV. Biomacromolecules 4:1433–1442
Hong Z, Zhang P, He C et al (2005) Nano-composite of poly(l-lactide) and surface grafted hydroxyapatite: mechanical properties and biocompatibility. Biomaterials 26:6296–6304
Huang L, McMillan RA, Apkarian RP et al (2000) Generation of synthetic elastin-mimetic small diameter fibers and fiber networks. Macromolecules 33:2989–2997
Huang L, Nagapudi K, Apkarian RP et al (2001) Engineered collagen: PEO nanofibers and fabrics. J Biomater Sci Polym Ed 12:979–993
Ishii D, Kinbara K, Ishida Y et al (2003) Chaperonin-mediated stabilization and ATP-triggered release of semiconductor nanoparticles. Nature 423:628–632
Itoh S, Kikuchi M, Takakuda K et al (2001) The biocompatibility and osteoconductive activity of a novel hydroxyapatite/collagen composite biomaterial, and its function as a carrier of rhBMP-2. J Biomed Mater Res A 54:445–453
Itoh S, Kikuchi M, Koyama Y et al (2004) Development of a hydroxyapatite/collagen nanocomposite as a medical device. Cell Transplant 13:451–461
Jain KK (2008) Regenerative medicine and tissue engineering. In: Jain KK (ed) The handbook of nanomedicine. Humana Press Inc., Totowa
Jaiswal JK, Mattoussi H, Mauro JM et al (2002) Long-term multiple color imaging of live cells using quantum dot bioconjugates. Nat Biotechnol 21:47–51
Jaleel MA, Tsai AC, Sarkar S et al (2004) Stromal cell-derived factor-1 (SDF-1) signalling regulates human placental trophoblast cell survival. Mol Hum Reprod 10:901–909
Jiang H, Hu Y, Li Y et al (2005) A facile technique to prepare biodegradable coaxial electrospun nanofibers for controlled release of bioactive agents. J Control Release 108:237–243
Josephson L, Tung C-H, Moore A et al (1999) High-efficiency intracellular magnetic labeling with novel superparamagnetic-tat peptide conjugates. Bioconjugate Chem 10:186–191
Khang D, Carpente J, Chun YW et al (2010) Nanotechnology for regenerative medicine. In: Biomed Microdevices 12:575–587
Kim J-s, Reneker DH (1999) Mechanical properties of composites using ultrafine electrospun fibers. Polym Composite 20:124–131
Kiritsy CP, Lynch SE (1993) Role of growth factors in cutaneous wound healing: a review. Crit Rev Oral Biol Med 4:729–760
Kisiday J, Jin M, Kurz B et al (2002) Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: implications for cartilage tissue repair. Proc Natl Acad Sci 99:9996–10001
Klopsch C, Furlani D, Gabel R et al (2009) Intracardiac injection of erythropoietin induces stem cell recruitment and improves cardiac functions in a rat myocardial infarction model. J Cell Mol Med 13:664–679

Koch AM, Reynolds F, Kircher MF et al (2003) Uptake and metabolism of a dual fluorochrome Tat-nanoparticle in HeLa cells. Bioconjugate Chem 14:1115–1121
Kohler N, Fryxell GE, Zhang M (2004) A bifunctional poly(ethylene glycol) silane immobilized on metallic oxide-based nanoparticles for conjugation with cell targeting agents. J Am Chem Soc 126:7206–7211
Kostura L, Kraitchman DL, Mackay AM et al (2004) Feridex labeling of mesenchymal stem cells inhibits chondrogenesis but not adipogenesis or osteogenesis. NMR Biomed 17:513–517
Kothapalli CR, Shaw MT, Wei M (2005) Biodegradable HA-PLA 3-D porous scaffolds: effect of nano-sized filler content on scaffold properties. Acta Biomater 1:653–662
Kuntz R, Saltzman W (1997) Neutrophil motility in extracellular matrix gels: mesh size and adhesion affect speed of migration. Biophys J 72:1472–1480
Kwon IK, Matsuda T (2005) Co-electrospun nanofiber fabrics of poly(l-lactide-co-ε-caprolactone) with type I collagen or heparin. Biomacromolecules 6:2096–2105
Kwon IK, Park KD, Choi SW et al (2001) Fibroblast culture on surface-modified poly (glycolide-co-ε-caprolactone) scaffold for soft tissue regeneration. J Biomater Sci Polym Ed 12:1147–1160
Kwon IK, Kidoaki S, Matsuda T (2005) Electrospun nano- to microfiber fabrics made of biodegradable copolyesters: structural characteristics, mechanical properties and cell adhesion potential. Biomaterials 26:3929–3939
Larson DR, Zipfel WR, Williams RM et al (2003) Water-soluble quantum dots for multiphoton fluorescence imaging in vivo. Science 300:1434–1436
Layrolle P, Daculsi G (2006) Nanostructured biomaterials. Nanomedicine 1:493–494
Lemon BI, Crooks RM (2000) Preparation and characterization of dendrimer-encapsulated CdS semiconductor quantum dots. J Am Chem Soc 122:12886–12887
Lewin M, Carlesso N, Tung C-H et al (2000) Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. Nat Biotechnol 18:410–414
Lewis JD, Destito G, Zijlstra A et al (2006) Viral nanoparticles as tools for intravital vascular imaging. Nat Med 12:354–360
Lewus KE, Nauman EA (2005) In vitro characterization of a bone marrow stem cell-seeded collagen gel composite for soft tissue grafts: effects of fiber number and serum concentration. Tissue Eng 11:1015–1022
Li W-J, Laurencin CT, Caterson EJ et al (2002) Electrospun nanofibrous structure: a novel scaffold for tissue engineering. J Biomed Mater Res A 60:613–621
Li M, Mondrinos MJ, Gandhi MR et al (2005) Electrospun protein fibers as matrices for tissue engineering. Biomaterials 26:5999–6008
Li W, Ma N, Ong LL et al (2007a) Bcl-2 engineered MSCs inhibited apoptosis and improved heart function. Stem Cells 25:2118–2127
Li W, Nesselmann C, Zhou Z et al (2007b) Gene delivery to the heart by magnetic nanobeads. J Magn Magn Mater 311:336–341
Li W, Ma N, Ong LL et al (2008) Enhanced thoracic gene delivery by magnetic nanobead-mediated vector. J Gene Med 10:897–909
Liao SS, Cui FZ, Zhang W et al (2004) Hierarchically biomimetic bone scaffold materials: nano-HA/collagen/PLA composite. J Biomed Mater Res B 68B:158–165
Lidke DS, Nagy P, Heintzmann R et al (2004) Quantum dot ligands provide new insights into erbB/HER receptor–mediated signal transduction. Nat Biotechnol 22:198–203
Liu H, Webster TJ (2007) Nanomedicine for implants: a review of studies and necessary experimental tools. Biomaterials 28:354–369
Luu YK, Kim K, Hsiao BS et al (2003) Development of a nanostructured DNA delivery scaffold via electrospinning of PLGA and PLA-PEG block copolymers. J Control Release 89:341–353
Ma L, Gao C, Mao Z et al (2003) Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. Biomaterials 24:4833–4841
Ma N, Stamm C, Kaminski A et al (2005a) Human cord blood cells induce angiogenesis following myocardial infarction in NOD/scid-mice. Cardiovasc Res 66:45–54
Ma Z, Kotaki M, Inai R et al (2005b) Potential of nanofiber matrix as tissue-engineering scaffolds. Tissue Eng 11:101–109

Ma Z, Gao C, Gong Y et al (2005c) Cartilage tissue engineering PLLA scaffold with surface immobilized collagen and basic fibroblast growth factor. Biomaterials 26:1253–1259
Ma L, Zhou J, Gao C et al (2007) Incorporation of basic fibroblast growth factor by a layer-by-layer assembly technique to produce bioactive substrates. J Biomed Mater Res B 83B:285–292
Mao Z, Ma L, Zhou J et al (2005) Bioactive thin film of acidic fibroblast growth factor fabricated by layer-by-layer assembly. Bioconjugate Chem 16:1316–1322
Martin GE, Cockshott ID (1977) A product comprising a mat of fibers prepared by electrostatically spinning an organic material and collecting the spun fibers on a suitable receiver. U.S. Patent 4043331
Mastrobattista E, van-der Aa MAEM, Hennink WE et al (2006) Artificial viruses: a nanotechnological approach to gene delivery. Nat Rev Drug Discov 5:115–121
Matthews JA, Wnek GE, Simpson DG et al (2002) Electrospinning of collagen nanofibers. Biomacromolecules 3:232–238
Matthews JA, Boland ED, Wnek GE et al (2003) Electrospinning of collagen type II: a feasibility study. J Bioact Compat Pol 18:125–134
Maysinger D, Behrendt M, Lalancette-Hébert M et al (2007) Real-time imaging of astrocyte response to quantum dots: in vivo screening model system for biocompatibility of nanoparticles. Nano Lett 7:2513–2520
McIntyre JO, Fingleton B, Wells KS et al (2004) Development of a novel fluorogenic proteolytic beacon for in vivo detection and imaging of tumour-associated matrix metalloproteinase-7 activity. Biochem J 377:617–628
McQuibban GA, Butler GS, Gong JH et al (2001) Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. J Biol Chem 276:43503–43508
Mikos AG, Thorsen AJ, Czerwonka LA et al (1994) Preparation and characterization of poly(L-lactic acid) foams. Polymer 35:1068–1077
Miller DC, Thapa A, Haberstroh KM et al (2004) Endothelial and vascular smooth muscle cell function on poly(lactic-co-glycolic acid) with nano-structured surface features. Biomaterials 25:53–61
Miller DC, Haberstroh KM, Webster TJ (2007) PLGA nanometer surface features manipulate fibronectin interactions for improved vascular cell adhesion. J Biomed Mater Res A 81A:678–684
Moghimi SM, Hunter AC (2000) Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. Trends Biotechnol 18:412–420
Mooney DJ, Baldwin DF, Suh NP et al (1996) Novel approach to fabricate porous sponges of poly(D, L-lactic-co-glycolic acid) without the use of organic solvents. Biomaterials 17:1417–1422
Mulder WJM, Koole R, Brandwijk RJ et al (2006) Quantum dots with a paramagnetic coating as a bimodal molecular imaging probe. Nano Lett 6:1–6
Murugan R, Ramakrishna S (2007) Design strategies of tissue engineering scaffolds with controlled fiber orientation. Tissue Eng 13:1845–1866
Nam YS, Park TG (1999a) Porous biodegradable polymeric scaffolds prepared by thermally induced phase separation. J Biomed Mater Res A 47:8–17
Nam YS, Park TG (1999b) Biodegradable polymeric microcellular foams by modified thermally induced phase separation method. Biomaterials 20:1783–1790
Nam YS, Yoon JJ, Park TG (2000) A novel fabrication method of macroporous biodegradable polymer scaffolds using gas foaming salt as a porogen additive. J Biomed Mater Res B 53:1–7
Niemeyer CM, Ceyhan B (2001) DNA-directed functionalization of colloidal gold with proteins. Angew Chem Int Ed 40:3685–3688
Papisov MI, Bogdanov A, Schaffer B et al (1993) Colloidal magnetic resonance contrast agents: effect of particle surface on biodistribution. J Magn Magn Mater 122:383–386
Parak WJ, Boudreau R, Le-Gros M et al (2002) Cell motility and metastatic potential studies based on quantum dot imaging of phagokinetic tracks. Adv Mater 14:882–885
Peterson JT, Li H, Dillon L et al (2000) Evolution of matrix metalloprotease and tissue inhibitor expression during heart failure progression in the infarcted rat. Cardiovasc Res 46:307–315

Ramachandran GN (1988) Stereochemistry of collagen. Int J Pept Proteins Res 31:1–16
Rao J, Dragulescu-Andrasi A, Yao H (2007) Fluorescence imaging in vivo: recent advances. Curr Opin Biotechnol 18:17–25
Reddy ST, Rehor A, Schmoekel HG et al (2006) In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. J Control Release 112:26–34
Reneker DH, Chun I (1996) Nanometre diameter fibres of polymer, produced by electrospinning. Nanotechnology 7:216–223
Rhyner MN, Smith AM, Gao X et al (2006) Quantum dots and multifunctional nanoparticles: new contrast agents for tumor imaging. Nanomedicine 1:209–217
Rogach A, Kershaw SV, Burt M et al (1999) Colloidally prepared HgTe nanocrystals with strong room-temperature infrared luminescence. Adv Mater 11:552–555
Rovira A, Bareille R, Lopez I et al (1993) Preliminary report on a new composite material made of calcium phosphate, elastin peptides and collagens. J Mater Sci Mater Med 4:372–380
Sasaki T, Iwasaki N, Kohno K et al (2008) Magnetic nanoparticles for improving cell invasion in tissue engineering. J Biomed Mater Res A 86A:969–978
Schaffer BK, Linker C, Papisov M et al (1993) MION-ASF: biokinetics of an MR receptor agent. Magn Reson Imaging 11:411–417
Schellenberger EA, Bogdanov AJ, Högemann D et al (2002) Annexin V-CLIO: a nanoparticle for detecting apoptosis by MRI. Mol Imaging 1:102–107
Schellenberger EA, Sosnovik D, Weissleder R et al (2004) Magneto/optical annexin V, a multimodal protein. Bioconjugate Chem 15:1062–1067
Shah BS, Clark PA, Moioli EK et al (2007) Labeling of mesenchymal stem cells by bioconjugated quantum dots. Nano Lett 7:3071–3079
Silva GA, Czeisler C, Niece KL et al (2004) Selective differentiation of neural progenitor cells by high-epitope density nanofibers. Science 303:1352–1355
Simon SR (1994) Orthopaedic basic science. American Academy of Orthopaedic Surgeons, Rosemont
So M-K, Xu C, Loening AM et al (2006) Self-illuminating quantum dot conjugates for in vivo imaging. Nat Biotechnol 24:339–343
Solanki A, Kim JD, Lee K-B (2008) Nanotechnology for regenerative medicine: nanomaterials for stem cell imaging. Nanomedicine 3:567–578
Soto CM, Blum AS, Vora GJ et al (2006) Fluorescent signal amplification of carbocyanine dyes using engineered viral nanoparticles. J Am Chem Soc 128:5184–5189
Stitzel J, Liu J, Lee SJ et al (2006) Controlled fabrication of a biological vascular substitute. Biomaterials 27:1088–1094
Strom SC, Michalopoulos G (1982) Collagen as a substrate for cell growth and differentiation. Method Enzymol 82A:544–555
Sun Z, Zussman E, Yarin AL et al (2003) Compound core-shell polymer nanofibers by co-electrospinning. Adv Mater 15:1929–1932
Sun C, Sze R, Zhang M (2006) Folic acid-PEG conjugated superparamagnetic nanoparticles for targeted cellular uptake and detection by MRI. J Biomed Mater Res A 78A:550–557
Tabata Y (2003) Tissue regeneration based on growth factor release. Tissue Eng 9:S5–S15
Tampieri A, Celotti G, Landi E et al (2003) Biologically inspired synthesis of bone-like composite: self-assembled collagen fibers/hydroxyapatite nanocrystals. J Biomed Mater Res A 67A:618–625
Taniguchi N (1974) On the basic concept of 'nano-technology'. In: Proceedings of the international conference of production engineering, Tokyo, pp 18–23
TenHuisen KS, Martin RI, Klimkiewicz M et al (1995) Formation and properties of a synthetic bone composite: hydroxyapatite-collagen. J Biomed Mater Res 29:803–810
Thomas V, Dean DR, Vohra YK (2006a) Nanostructured biomaterials for regenerative medicine. Curr Nanosci 2:155–177
Thomas V, Jagani S, Johnson K et al (2006b) Electrospun bioactive nanocomposite scaffolds of polycaprolactone and nanohydroxyapatite for bone tissue engineering. J Nanosci Nanotechnol 6:487–493

Thomas V, Dean DR, Jose MV et al (2007) Nanostructured biocomposite scaffolds based on collagen co-electrospun with nanohydroxyapatite. Biomacromolecules 8:631–637
Thorek DLJ, Chen AK, Czupryna J et al (2006) Superparamagnetic iron oxide nanoparticle probes for molecular imaging. Ann Biomed Eng 34:23–38
Vaccaro DE, Yang M, Weinberg JS et al (2008) Cell tracking using nanoparticles. J Cardiovasc Trans Res 1:217–220
van Tilborg GAF, Mulder WJM, Chin PTK et al (2006) Annexin A5-conjugated quantum dots with a paramagnetic lipidic coating for the multimodal detection of apoptotic cells. Bioconjugate Chem 17:865–868
van Wachem PB, Plantinga JA, Wissink MJ et al (2001) In vivo biocompatibility of carbodiimide-crosslinked collagen matrices: effects of crosslink density, heparin immobilization, and bFGF loading. J Biomed Mater Res 55:368–378
Venugopal J, Ma LL, Yong T et al (2005) In vitro study of smooth muscle cells on polycaprolactone and collagen nanofibrous matrices. Cell Biol Int 29:861–867
Verreck G, Chun I, Rosenblatt J et al (2003) Incorporation of drugs in an amorphous state into electrospun nanofibers composed of a water-insoluble, nonbiodegradable polymer. J Control Release 92:349–360
Wang W, Li W, Ong LL et al (2009) Localized and sustained SDF-1 gene release mediated by fibronectin films: a potential method for recruiting stem cells. Int J Artif Organs 32:141–149
Wang W, Li W, Ong LL et al (2010) Localized SDF-1alpha gene release mediated by collagen substrate induces CD117+ stem cell homing. J Cell Mol Med 14:392–402
Webster TJ (2001) Nanophase ceramics: the future orthopedic and dental implant material. In: Ying JY-R (ed) Nanostructured materials. Academic Press, New York
Webster TJ, Ejiofor JU (2004) Increased osteoblast adhesion on nanophase metals: Ti, Ti6Al4V, and CoCrMo. Biomaterials 25:4731–4739
Webster TJ, Siegel RW, Bizios R (1999) Osteoblast adhesion on nanophase ceramics. Biomaterials 20:1221–1227
Wei G, Ma PX (2006) Macroporous and nanofibrous polymer scaffolds and polymer/bone-like apatite composite scaffolds generated by sugar spheres. J Biomed Mater Res A 78A:306–315
Wei G, Ma PX (2008) Nanostructured biomaterials for regeneration. Adv Funct Mater 18:3568–3582
Wei G, Jin Q, Giannobile WV et al (2006) Nano-fibrous scaffold for controlled delivery of recombinant human PDGF-BB. J Control Release 112:103–110
Weissleder R, Papisov M (1992) Pharmaceutical iron oxides for MR imaging. Rev Magn Reson Med 4:1–20
Weissleder R, Bogdanov A, Neuwelt EA et al (1995) Long-circulating iron oxides for MR imaging. Adv Drug Deliv Rev 16:321–334
Wentworth B, Stewart J, Westrich J et al (2007) Studies on the retention of cells delivered to the rat heart. Paper presented at: European Society of Cardiology Congress, Vienna, Austria
Whitesides GM, Grzybowski B (2002) Self-assembly at all scales. Science 295:2418–2421
Winter JO, Liu TY, Korgel BA et al (2001) Recognition molecule directed interfacing between semiconductor quantum dots and nerve cells. Adv Mater 13:1673–1677
Wnek GE, Carr ME, Simpson DG et al (2003) Electrospinning of nanofiber fibrinogen structures. Nano Lett 3:213–216
Wu C, Barnhill H, Liang X et al (2005) A new probe using hybrid virus-dye nanoparticles for near-infrared fluorescence tomography. Optics Comm 255:366–374
Yang F, Murugan R, Ramakrishna S et al (2004a) Fabrication of nano-structured porous PLLA scaffold intended for nerve tissue engineering. Biomaterials 25:1891–1900
Yang XB, Bhatnagar RS, Li S et al (2004b) Biomimetic collagen scaffolds for human bone cell growth and differentiation. Tissue Eng 10:1148–1159
Yao C, Slamovich EB, Webster TJ (2008) Enhanced osteoblast functions on anodized titanium with nanotube-like structures. J Biomed Mater Res A 85A:157–166
Ye L, Huang X (2005) MAP2: multiple alignment of syntenic genomic sequences. Nucl Acids Res 33:162–170

Yoshimoto H, Shin YM, Terai H et al (2003) A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering. Biomaterials 24:2077–2082
Yu Y-C, Tirrell M, Fields GB (1998) Minimal lipidation stabilizes protein-like molecular architecture. J Am Chem Soc 120:9979–9987
Yu Y-C, Roontga V, Daragan VA et al (1999) Structure and dynamics of peptide-amphiphiles incorporating triple-helical proteinlike molecular architecture. Biochemistry 38:1659–1668
Yu WW, Qu L, Guo W et al (2003) Experimental determination of the extinction coefficient of CdTe, CdSe, and CdS nanocrystals. Chem Mater 15:2854–2860
Zaheer A, Lenkinski RE, Mahmood A et al (2001) In vivo near-infrared fluorescence imaging of osteoblastic activity. Nat Biotechnol 19:1148–1154
Zeng J, Chen X, Xu X et al (2003) Ultrafine fibers electrospun from biodegradable polymers. J Appl Polym Sci 89:1085–1092
Zhang S (2003) Fabrication of novel biomaterials through molecular self-assembly. Nat Biotechnol 21:1171–1178
Zhang R, Ma PX (1999) Poly(α-hydroxyl acids)/hydroxyapatite porous composites for bone-tissue engineering. I. Preparation and morphology. J Biomed Mater Res A 44:446–455
Zhang R, Ma PX (2000) Synthetic nano-fibrillar extracellular matrices with predesigned macroporous architectures. J Biomed Mater Res A 52:430–438
Zhang L, Webster TJ (2009) Nanotechnology and nanomaterials: promises for improved tissue regeneration. Nano Today 4:66–80
Zhang Y, Huang Z-M, Xu X et al (2004) Preparation of core–shell structured PCL-r-gelatin bi-component nanofibers by coaxial electrospinning. Chem Mater 16:3406–3409
Zhang Y, Ouyang H, Lim CT et al (2005a) Electrospinning of gelatin fibers and gelatin/PCL composite fibrous scaffolds. J Biomed Mater Res B 72B:156–165
Zhang YZ, Venugopal J, Huang Z-M et al (2005b) Characterization of the surface biocompatibility of the electrospun PCL-collagen nanofibers using fibroblasts. Biomacromolecules 6:2583–2589
Zhou Q, Gong Y, Gao C (2005) Microstructure and mechanical properties of poly(L-lactide) scaffolds fabricated by gelatin particle leaching method. J Appl Polym Sci 98:1373–1379
Zong X, Kim K, Fang D et al (2002) Structure and process relationship of electrospun bioabsorbable nanofiber membranes. Polymer 43:4403–4412

Chapter 24
Biointerface Technology

Joachim Rychly

Abstract The application of biomaterials to regenerate tissues requires research of the interface between the synthetic material and the living tissue. Because biomaterials represent a synthetic extracellular matrix that controls the cell biology by mechanism of cell adhesion, basic mechanisms of cell adhesion are addressed. The technology of designing instructive materials involves chemical modifications by grafting of chemical groups, adhesion ligands and growth factors. Physical characteristics of the materials are created by modifications of the surfaces structure and stiffness of the material. Because stem cells have emerged as promising cells to address the challenge of tissue regeneration the control of stem cells by the characteristic of materials is discussed. Insights into the mechanism at the biointerface that are involved in the regulation of stem cells by materials will advance the development of innovative biomaterials in regenerative medicine.

24.1 Introduction: An Historical Perspective

The biointerface is the interface between a nonviable material and the biological tissue or a cell. Mechanisms of the interaction between a material and the biological tissue control the reaction of the tissue and may also determine the fate of the material. The application of materials as medical implants or prostheses has a more than 2,000 years history. To replace limbs, eyes, teeth, part of the skull or bone, beside wood or ivory the ancient cultures used mostly different metals. The first polymer as an implant was introduced by the British ophthalmologist Harold Ridley in 1949,

J. Rychly (✉)
Laboratory of Cell Biology, University of Rostock,
Schillingallee 69, Rostock 18057, Germany
e-mail: joachim.rychly@med.uni-rostock.de

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_24,

when he used poly (methyl methacrylate) to replace a cataracted lens of a patient (Ridley 1952). He made the observation that the eyes of pilots who had shards of canopy plastic in their eyes due to enemy machine gun fire, tolerated this material, without ongoing reactions. In addition to implants, also ex vivo devices, like dialysis equipments or heart lung machines form a biointerface, in that case mostly with cells of the blood.

With the introduction of hip implants, vascular grafts or the kidney dialysis first principles of application of medical materials were given by the late 1960s. The principal demand for a medical material was that the interaction of the material with the biological system should not provoke harmful reactions. The term "biocompatibility" originally refers to material characteristics of having no toxic effects or inducing mutagenesis and inflammation. The goal of the early biomaterials was to achieve a biological "inertness". The challenge of the new generation of materials is to create bioactive surfaces that are suitable to specifically control the biology of the tissue. In the field of regenerative medicine the control of stem cell plays a principal role. Therefore, the designing of implant materials is focussed on the question how characteristics of the materials are able to steer all the biological functions of a stem cell, which include self-renewal, differentiation to a specific cellular phenotype, secretion of bioactive factors, or migration. The development of such bioactive material surfaces requires the interdisciplinary collaboration between disciplines of engineering and the life sciences. The progress in this field depends on both the understanding of the biological mechanisms and the development of technological methods. The driving force for the design of bioactive material surfaces is the understanding of the complex mechanisms on the cellular level that determine the regenerative processes in the different tissues of the organism. Therefore, in this chapter first a review of cell biological mechanisms will be given with a focus on the adhesive interactions of cells with the extracellular matrix. These interactions play a key role at the cell-material interface and basically, the aim of material design is to control the cell biology by modifications of the chemical and physical properties of the material surfaces.

24.2 Background/Principles

24.2.1 Mechanisms of Cell Adhesion

Cells are regulated by different signals induced by soluble factors, cell-cell contacts and the interaction of cells with the extracellular matrix. Proteins of the extracellular matrix, like collagens, fibronectin, laminin, elastin are secreted by cells and differ in their composition depending on the type of tissue. For example, collagen I is a characteristic matrix component for bone, collagen II for cartilage or laminin for the basal membrane of the epithelium and endothelium. The composition and structure of the extracellular matrix is dynamic and vary which determine its function. This is obvious during processes of the development and tissue differentiation. For example,

during the development of branched organs like mammary gland, kidney, gut and lung the branched units are surrounded by a microenvironment that change in composition and spatial distribution over the time (Rozario and Desimone 2010). The spatio-temporal expression and deposition of extracellular matrix provides instructive differentiation signals. In the mouse development, myogenic differentiation occurs as laminin, collagen IV and entactin expression increases, whereas fibronectin expression decreases (Godfrey and Gradall 1998). Although the control of stem cell differentiation by the extracellullar matrix appears complex, defined matrix molecules induced specific differentiation of stem cells. Embryonic stem cells are normally not competent to differentiate to trophoblastic cells, however on collagen IV but not on laminin, fibronectin or collagen I the cells developed to a trophoblastic lineage (Schenke-Layland et al. 2007). Also directed differentiation of multipotent adult stem cells was dependent on the type of matrix protein. Neural stem cells developed to neurons, astrocytes and glia cells on laminin but not on fibronectin (Flanagan et al. 2006). Osteogenic differentiation of human mesenchymal stem cells was induced on laminin-5, collagen I and vitronectin (Klees et al. 2005; Kundu and Putnam 2006; Salasznyk et al. 2004). The studies also revealed that differentiation to the same phenotype might be differentially regulated by different matrix proteins (Kundu and Putnam 2006). As already mentioned, the extracellular matrix is a highly dynamic structure, which is constantly undergoing remodelling, i.e. assembly and degradation. Experiments using fluorescence time lapse-imaging demonstrated that in a cell culture individual fibrils of fibronectin were stretched and displaced (Sivakumar et al. 2006). Motile osteoblasts actively mediated fibronectin assembly by adding globules of matrix molecules to existing fibronectin fibrils and reorganized the extracellular matrix by shunting matrix material from one location to another or exchanged fibrillar material between fibrils. Remodelling of the extracellular matrix is the result of multiple processes, which requires at least two events: synthesis and proteolytic degradation of the components (Daley et al. 2008). Among the proteolytic enzymes, matrix metalloproteinases (MMPs) play a dominant role in the degradation of the extracellular matrix. Although matrix protein degradation remains a principal physiological function of MMPs, there is evidence that also other substrates, like peptide growth factors, tyrosine kinase receptors, chemokines are a target of MMPs, which indicates a more extensive involvement of MMPs in a variety of physiological processes (Page-McCaw et al. 2007; Stamenkovic 2003). The interaction of cells with the extracellular matrix is mediated by receptors of the integrin family which enable a bidirectional signal transduction (Hynes 2002; Takada et al. 2007). Integrins function as heterodimeric transmembrane receptors consisting of one ß and one α-subunit. In human, 18 α-subunits and 8 ß-subunits are described, which form at least 24 different receptors (van der Flier and Sonnenberg 2001; Wehrle-Haller and Imhof 2003). The combination of the ß with the α-subunit determines the binding specificity for the ECM ligand and a simplified classification into three classes yields a group of integrins, which binds to the RGD sequence (amino acids Arg-Gly-Asp) of fibronectin or vitronectin, receptors which bind to laminin and integrins that bind to collagens (Wiesner et al. 2005). Activation of integrins which induces signal transduction involves conformational changes in the extracellular domain to expose

the ligand-binding site (Luo et al. 2007). The conformational changes also enable an increased binding avidity which leads to a clustering of hundreds or thousands integrin interactions with matrix ligands into tightly bound adhesive units (Legate et al. 2009). To connect integrins with the actin cytoskeleton in integrin mediated signal transduction, the formation of adhesion complexes at the interface between cell and substrate plays a dominant role. In these focal adhesions 157 molecules have been identified that are assembled in a "integrin adhesome" and enables signal transduction (Zaidel-Bar et al. 2007). Upon integrin binding to a ligand focal adhesions mature. First nascent adhesions are organized within the lamellipodium. During maturation the adhesions grow into dot-like structures, which then become elongated to form fibrillar adhesions (Geiger et al. 2001; Wehrle-Haller and Imhof 2002; Zaidel-Bar et al. 2003). This process is facilitated by the α-actinin-actin structures and requires myosin II (Choi et al. 2008). The functions of some of the numerous proteins assembled in focal adhesions have been elucidated. For example, talin facilitates the interaction of integrins with the cytoskeleton by direct binding to the integrin tail, or vinculin plays a role in the formation and growth of focal adhesions (Gallant et al. 2005; Humphries et al. 2007; Zhang et al. 2008). FAK appears to be responsible for turnover of focal adhesions and actin polymerization and is a major component in further downstream signalling events (Zhao and Guan 2009). Downstream, integrin signalling shares common pathways of growth factor receptors, like activation of MAP-kinases (Miyamoto et al. 1996; Moro et al. 1998). Beside the cross-talk between integrins and growth factor receptor pathways, also the physical proximity and lateral collaboration at the cell membrane between integrins and growth factor receptors are important to induce signaling and in consequence a biological function (Schneller et al. 1997).

24.2.2 Cellular Mechanotransduction

Cells are able to sense mechanical forces, which control their physiological functions. Physical forces act or are generated at the interface between the cell and the extracellular matrix (Geiger et al. 2009; Mammoto and Ingber 2009; Puklin-Faucher and Sheetz 2009). Therefore, the cellular components that facilitate cell adhesion to the extracellular matrix have a primary role in the cellular sensory machinery and are able to integrate and transduce mechanical signals. Transduction of mechanical forces is bidirectional. While cells are able to sense forces from outside they also generate forces to the extracellular matrix, which is facilitated by the cytoskeleton and regulated for example by actin polymerization (Galbraith et al. 2007; Giannone et al. 2007; Ingber 2006; Kumar et al. 2006). Myosin II is responsible for the contractile nature of the stress fibres to exert forces to the extracellular matrix (Katoh et al. 2001; Peterson et al. 2004). Integrins function as primary sensor and mechanotransducers and facilitate the mechanical coupling between inside and outside the cell (Schober et al. 2007; Wang et al. 1993). Transition of the β integrin subunit from an inactive state to an active conformation can be induced by mechanical

forces (Cluzel et al. 2005; Kim et al. 2004; Puklin-Faucher et al. 2006). Mechanical forces directly applied to integrins induce an accumulation of focal adhesion molecules and a direct physical link to the cytoskeleton by immobilizing of signalling proteins, like FAK to the actin cytoskeleton (Cox et al. 2006; Michael et al. 2009; Riveline et al. 2001; Schmidt et al. 1998). To convert mechanical forces into biochemical signalling events, proteins at the adhesive interface are stretched and expose binding sites (Vogel and Sheetz 2009). Vinculin binds to talin rod due to mechanically stretching of the talin molecule (del Rio et al. 2009). Recently, filamin A has been identified as a mechanotransductive substrate within the cytoskeleton. When strain is applied, β integrin binding to filamin A increased which enables its cytoskeletal anchorage, whereas the protein FilGAP dissociates from filamin A (Ehrlicher et al. 2011). Detailed studies revealed that fibrillar fibronectin can be extended by stretch more than eight-fold and the mechanically induced unfolding of fibrillar fibronectin alter the displayed binding sites (Klotzsch et al. 2009; Vogel 2006). Fibronectin contains different recognition sites for binding of serum proteins, other matrix proteins, cell adhesion proteins distributed over more than 54 domains that can be switched on and off be mechanical forces (Vogel and Sheetz 2009). Interestingly, the mechanical properties of the fibronectin fibres are regulated, old fibres become more unfolded with age than newly deposited fibres. Further, due to differences in the mechanical strain, fibrillar fibronectin is more unfolded on rigid than on soft substrates (Antia et al. 2008). In addition to a mechano-biochemical conversion near the adhesion site, there is evidence that cells are able to transduce mechanical signals directly to the nucleus because of a structural connectivity between extracellular matrix and cell nucleus (Maniotis et al. 1997; Wang et al. 2009). In this model, the cell is a "hard wired" tensegrity network which refers to a stable interconnected cytoskeleton that resists mechanical stresses and maintain shape stability (Ingber 1997; Stamenovic et al. 1996). The connection between cytoskeletal filaments and the nuclear membrane is facilitated by a LINC complex (linker of nucleoskeleton and cytoskeleton) containing nesprins, sun and lamin proteins (Crisp et al. 2006; Haque et al. 2006). Through lamin A, which binds transcription factors, mechanical forces could directly alter gene expression in the nucleus (Dechat et al. 2008). In addition, mechanically induced expansion or contraction of nuclear pores may alter transport processes into the nucleus (Feldherr and Akin 1990). Such direct force transmission between cell membrane and nucleus may induce a fast induction of gene expression and may explain a rapid increase of calcium in the nucleus (Pommerenke et al. 2002).

24.2.3 Interaction with the Extracellular Matrix in the Stem Cell Niche

The stem cell niche is a specialized microenvironment in various organs which provides an anatomical compartment to maintain a pool of stem cells (Jones and Wagers 2008). The microenvironment, which involves soluble factors, the interaction

with other cells and an extracellular matrix, regulate stemness, survival and migration out of the niche (Kolf et al. 2007). To mimic the mechanisms in a niche by bioactive material surfaces, the extracellular matrix is of primary interest. Evidence exists that the composition and mechanical properties of extracellular matrix determines the fate of stem cells in a niche (Daley et al. 2008). It became further obvious that the dynamic remodelling of the extracellular matrix at a specific time and in a tissue-specific manner within a niche function as important switch to trigger stem cell differentiation or mobilization. However, detailed information about a precise role of the extracellular matrix in a niche are rare. Differential expression of integrin-β1 has been observed to regulate cell restriction and mobility of stem cells in the epidermal stem cell niche (Jensen et al. 1999). The fate of neuronal stem cells appeared to be dependent on the expression of β1-integrin (Yoshida et al. 2003). Neuronal stem cell differentiation was accompanied by a decrease in α5β1-Integrin. In a hematopoietic stem cell niche, the matrix glycoptrotein osteopontin plays a role for the hematopoietic stem cells to localize at the endosteal bone surface (Nilsson et al. 2005). In addition, osteopontin was found to suppress hematopoietic stem cell proliferation. Recent studies stressed the assumption that the type of extracellular matrix may determine the direction of stem cell differentiation. Mesenchymal stem cells are localized in a perivascular niche and are exposed to signals from vascular cells (Crisan et al. 2008). On extracellular matrix derived from endothelial cells, mesenchymal stem cells developed markers of endothelial or smooth muscle cells (Lozito et al. 2009).

24.3 Technological and Biological Opportunities for Therapeutic Devices

24.3.1 Chemical Modification to Control the Biointerface

24.3.1.1 Modification of Chemical Groups

Chemical as well as physical characteristics of a material control the biological response of the tissue. For tissue regeneration, the key question is that, how the properties of a biomaterial specifically control the different biological functions of stem cells. Different steps of surface designing can generate a bioactive chemistry of a material. First, the chemistry is determined by the pure uncoated material. Next, the chemistry can be modified by grafting chemical groups on the surface, which alter the surface charge and the wettability. More specifically, molecules of the extracellular matrix or peptides which are characteristic of matrix domains and function as binding sites may be immobilized. Last, soluble factors, like growth factor may be incorporated into the material surface, which might by active as solid-phase ligand or which could released by various mechanisms.

Dependent on the application regarding the tissue and function, materials for implants reach from metals to synthetic polymers and natural materials. All these materials differ in the chemistry of the surface. At the interface to a material surface the interaction of the cell is mediated by extracellular matrix proteins. However, prior to a matrix production of the cell, a first adhesive contact of the cell to the substrate can be mediated by a hyaluronan coat of the cell (Cohen et al. 2006; Evanko et al. 2007). The strength of this interaction differs in dependence on the material to which the cell does adhere (Finke et al. 2007). For the subsequent integrin mediated adhesion, adsorption and organization of the extracellular matrix proteins to a material are required. The role of chemical variations of the surface to mediate adhesion dependent stimulation of biological functions of stem cells can be evaluated by generating polymers with different combinations of monomers. Combining 25 different monomers of acrylates to generate 576 polymers allowed a screening to identify materials with the ability to stimulate proliferation and differentiation of human embryonic stem cells (Anderson et al. 2004). Some of the polymers allowed for a high level of cytokeratin positive cells, indicating differentiation to epithelial cells. Interestingly, for some materials proliferation was observed only in the absence of retinoic acid as a soluble factor. This indicates an interaction of signals from soluble factors and the adhesive substrate. A relationship was also established between the ability of the polymers to adsorb fibronectin and cell adhesion (Keselowsky et al. 2003; Mei et al. 2009). Polymers are not only capable to generate different amounts of adsorbed fibronectin, but also induce different activities of fibronectin (Mei et al. 2009). Different techniques have been used to modify the chemistry of a material surface, which involved the use of self assembled monolayers of alkanethiols, silanisation, plasma treatment, radiation grafting (Curran et al. 2005; Keselowsky et al. 2005; Ratner 1995). Grafting of functional groups using glow discharge plasma deposition was also successfully applied to modify titanium surfaces (Nebe et al. 2007). A major challenge of these modifications is the precise control of functional groups. The spectrum of functional groups comprises amino, methyl, hydroxyl, ether, carbonyl, carboxyl and carbonate. Specific alterations of the chemistry were found to guide differentiation and proliferation of mesenchymal stem cells (Curran et al. 2006; Phillips et al. 2010). $–NH_2$ and –SH modified surfaces stimulated osteogenic differentiation, whereas –OH and –COOH modified surfaces promoted chondrogenesis. Under specific culture conditions, $-NH_2$ surfaces enhanced the formation of adipogenic cells (Phillips et al. 2010). Generation of $–CH_3$ groups maintained the phenotype of mesenchymal stem cells (Curran et al. 2006). These biological responses of the cells depend on mechanisms related to changes in the cell-extracellular matrix interaction. Surface chemistry of a material can induce changes in the conformation of fibronectin, which modifies binding of integrins and induces short-term changes in focal adhesion formation (Keselowsky et al. 2004). Generation of $–NH_2$ groups on titanium surfaces using plasma polymerized allyl amine promoted the spreading of osteoblasts (Nebe et al. 2007). Titanium implants are widely used as bone substitutes, e. g. for artificial hip or knee joints. To stimulate bone regeneration at the interface to the bone tissue, titanium coating with calcium phosphate is a suitable approach because of the

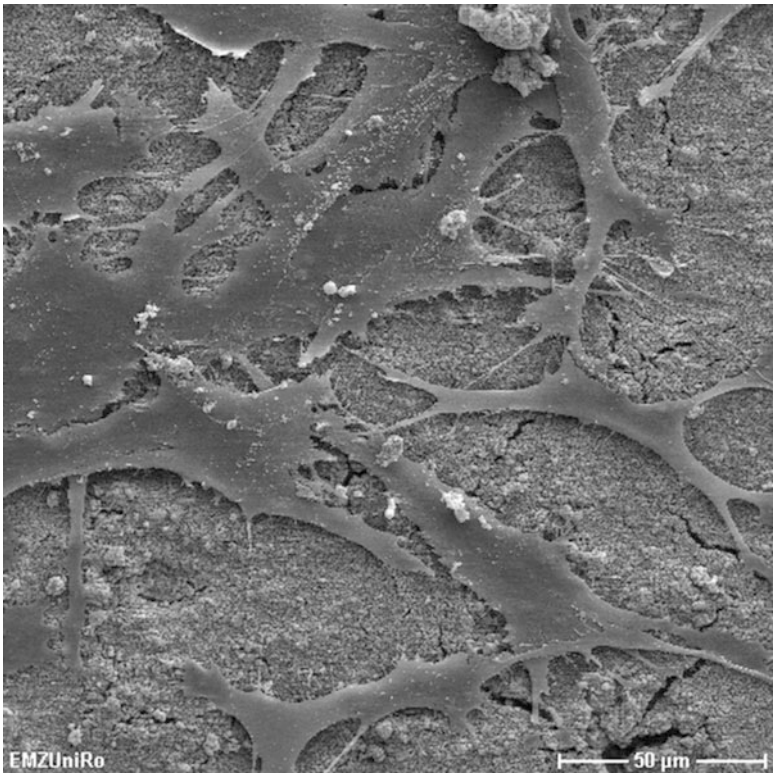


Fig. 24.1 Mesenchymal stem cells adhere, spread and form a flat morphology on hydroxyapatite coated surfaces

similarity with the mineral phase present in bone (de Groot et al. 1998; de Jonge et al. 2008). Similarly, calcium phosphate composites are applied as degradable scaffolds to heal bone defects (El-Ghannam 2005). The most successful technique to coat metallic implant with calcium phosphate has been the plasma-spray technique. Because coating must be at least 50 µm thick to completely cover the surface other methods including sol-gel deposition, electrospray deposition, electrolytic deposition have been applied and each has its advantages and disadvantages (de Jonge et al. 2008). Calcium phosphate coatings are described to induce an increased bone-to-implant contact and therefore are regarded as osteoconductive (Barrere et al. 2003; Leeuwenburgh et al. 2006). To see, whether calcium phosphate surfaces may affect bone regeneration, a number of in vitro studies demonstrated that calcium phosphate promote the osteogenic differentiation of mesenchymal stem cells (Cordonnier et al. 2010; Moreau and Xu 2009; Muller et al. 2008; Sun et al. 2008). Although the mechanisms are not known, the observed strong adsorption of fibronectin and vitronectin, as well as a very flat morphology of stem cells on a calcium phosphate surface (Fig. 24.1) could support an osteogenic differentiation (Kilpadi et al. 2001; Walschus et al. 2009).

24.3.1.2 Grafting of Cell Adhesion Ligands

To further specifically control cell adhesion, material surfaces can be grafted with complete molecules of the extracellular matrix or synthetic peptide sequences which represent binding sites of matrix proteins. The best known of these is the RGD peptide containing the amino acids arginine, glycine, aspartic acid which is found in fibronectin, laminin, collagen type IV, tenascin and thrombospondin (Benoit and Anseth 2005; Comisar et al. 2007) and several other adhesion molecules. Structural modifications of the peptides from linear to cyclic RGD peptides are potent alternatives and can enhance affinity towards a receptor or stimulate cell adhesion (Durrieu et al. 2004; Maeda et al. 1994). In most cases RGD peptides are linked to polymers via stable covalent amide bonds. In this case an activated surface carboxylic acid group reacts with the nucleophilic N-terminus of the peptide (Lin et al. 1994). Alternatively, a coupling is possible in a two step protocol. First, the surface carboxyl group is activated as an ester and followed by coupling the peptide in water (Jo et al. 2000). Beside synthetic polymers, other materials, including natural polymers, starch, dextran and inorganic materials have been coated with RGD peptides (Hersel et al. 2003). Among the inorganic materials, titanium and hydroxylapatite were successfully coated with RGD peptides (Fujisawa et al. 1997; Itoh et al. 2002; Rezania et al. 1999). On hydroxylapatite, RGD-peptides were immobilized via negatively charged anchoring groups, like glutamic acid, phosphonates or natural HA-binding amino acid sequences (Gilbert et al. 2000; Hersel et al. 2003; Itoh et al. 2002). To prevent unspecific protein adsorption, grafting of RGD peptides can be combined with passivation of the material surface using e.g. poly(ethylene glycol) (Banerjee et al. 2000; Drumheller and Hubbell 1995). Star-shaped poly (ethylene glycol) prepolymers were used to prevent unspecific protein adsorption and allowed the binding of RGD peptides for specific adhesion of mesenchymal stem cells (Groll et al. 2005). Cell experiments on materials coated with matrix proteins or peptides revealed that integrin mediated interactions with the substrate are complex and require flexible and dynamic mechanisms. Therefore, the introduction of a spacer to bind RGD peptides or matrix proteins improved cell attachment (Craig et al. 1995; Kantlehner et al. 2000). When collagen was immobilized to a polyether ether ketone via glutardialdehyde, osteoblasts did adhere but spread only when polyethylene glycol as spacer was introduced (Fig. 24.2). To further enable a dynamic interaction of cells with the adhesive substrate and remodel the extracellular matrix, materials were crosslinked by enzyme-degradable peptide sequences. The combination of integrin binding and matrix degradation by cellular metalloproteinases allowed the cells to migrate through a gel, which mimics tissue remodelling (Lutolf et al. 2003a). Enzymatically mediated cell migration has been provided using materials from chemically cross-linked hyaluronic acid (Bulpitt and Aeschlimann 1999; Park et al. 2003). Further, elastase-sensitive sequences were generated by crosslinking elastin-like units which contained the adhesion motif REDV (Girotti et al. 2004). Cleavage of the polymer yielded a bioactive VGVAPG fragment which stimulated cell proliferation. This functionality mimics dynamic processes of the extracellular matrix in vivo, whereby enzymic activities can liberate cryptic binding sites. Although

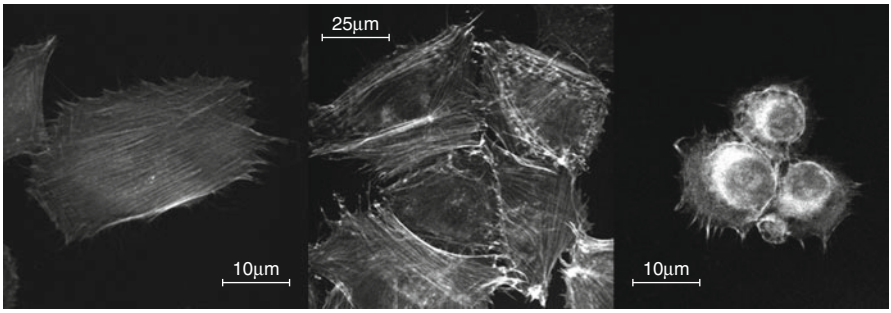


Fig. 24.2 The mode of collagen immobilization determines the spreading of osteoblasts: *Left*: On cover glass, which was coated by collagen adsorption, cells spread and form actin fibres; *middle*: Cells spread and form actin fibres on a polyether ether ketone (PEEK) surface coated with collagen, which was immobilized by glutardialdehyde (GDA) and polyethylenglycol was introduced as a spacer; *right*: Cells adhere but remain round without formation of actin fibres on PEEK, coated with collagen, immobilized via GDA alone

immobilization of matrix-derived peptides demonstrated support of cell adhesion, data of the biological specificity of such approaches are rare (Carson and Barker 2009). When titanium was passivated and grafted with the fibronectin fragment $FNIII_{7–10}$, this surface enhanced the osteogenic differentiation of mesenchymal stem cells relative to RGD immobilized surfaces (Petrie et al. 2008). This appeared to result from the specific targeting of the β1α5-integrin. The presentation of adhesion peptides in a structural organization that mimic fibrils of the extracellular matrix could further contribute to the biological outcome. RGD peptides in 3D-network of nanofibers promoted the osteogenic differentiation of mesenchymal stem cells (Hosseinkhani et al. 2006). In a three dimensional network of nanofibers the immobilization of the laminin epitope IKVAV induced the differentiation of neural progenitor cells into neurons (Silva et al. 2004).

24.3.1.3 Immobilization of Soluble Factors

The extracellular matrix provides a reservoir for growth factors, which can be released and act as soluble ligands (Hynes 2009). Evidence exists that also matrix-bound growth factors stimulate cell functions via solid-phase signals (Wijelath et al. 2006). Specific binding sites have been detected in the extracellular matrix which can regulate the function of growth factors (Hynes 2009). Therefore, the immobilization of growth factors and other bioactive molecules plays a role in the strategies of designing the surface of implant materials for tissue regeneration (Cartmell 2009; Lee and Shin 2007; Silva et al. 2009). Growth factors bound to biomaterial surfaces may have enhanced activities compared with a soluble form of the factor, as it has been shown for TGF-β1 covalently linked to a polymer and stimulating matrix production (Mann et al. 2001). Different techniques have been applied to tether and control the release of bioactive factors (Place et al. 2009). The easiest way to add

soluble factors is to load them into polymer matrix or to adsorb onto a composite (Soriano and Evora 2000; Ziegler et al. 2002). A variety of growth factors have been incorporated into hydrogels during the formation of the material in aqueous solution (Kanematsu et al. 2004). To tune the release of soluble proteins, the cross-linking density of the polymer can be modified (Hiemstra et al. 2007). bFGF could be released quantitatively from such hydrogels in 28 days. These techniques basically rely on the passive diffusion of growth factors from the matrix. Another strategy for protein release relies on a mechanical-responsive system (Augst et al. 2006; Lee and Mooney 2001). Many tissues, such as vasculature and musculature are mechanically dynamic. Mechanical compression could release factors from a material. Using a VEGF-containing alginate-hydrogel, it was shown that exposing mechanical strain to the hydrogel increased the release of VEGF (Lee et al. 2000). After implantation in mice, this mechanically induced release increased collateral vessel formation. Adding growth factors to ceramic materials is very convenient, because ceramics have a high affinity for proteins (Ziegler et al. 2002). Growth factors, such as TGF, FGF and VEGF were loaded to ceramics just by adsorption. The release patterns of most loaded ceramics seem to consist of an initial burst release of not bound protein followed by a second release dependent on the material/protein interaction (Habraken et al. 2007). Loading of calcium phosphate cements with growth factors was performed just by adding the protein to the liquid hardener, thereby distributing it equally through the cement. Bovine serum albumin can be used as carrier solution for growth factors to control the release of factors from the cement (Blom et al. 2002; Ruhe et al. 2006). Several in vivo studies proved the beneficial effects of growth factor loaded calcium phosphate scaffolds (Jansen et al. 2005; Kroese-Deutman et al. 2005; Ruhe et al. 2004; Seeherman and Wozney 2005).

More precise, growth factors can be immobilized to a material surface by covalent binding. This can be achieved by reacting of the side chains of polymers with amino acids of a growth factor. Several growth factors have been covalently linked to polyethylene glycol, including TGF, EGF, bFGF (Bentz et al. 1998; DeLong et al. 2005; Kuhl and Griffith-Cima 1996). To control the release of covalently attached growth factors by the cells, synthetic hydrogels have been generated which contained protease sensitive binding sites (Lutolf et al. 2003a; Zisch et al. 2003b). In this case the hydrogels are prepared with functionalities of natural extracellular matrix, i.e. the ability to mediate adhesion and to respond to proteolytic degradation by enzymes, such as metalloproteinases which are secreted by cells. As structural building blocks, end-functionalized polyethylene vinylsulfone chains were used with thiol-bearing peptides. Cross-linking occurred by incorporation of bis-cysteine peptides, which can be cleaved by proteases. Growth factors, like VEGF and BMP were bound to these structures and could be delivered on cell demand (Lutolf et al. 2003b; Zisch et al. 2003a). Using this approach, an active liberation of VEGF was confirmed which resulted in a remodelled vascularized tissue, when the matrix was implanted subcutaneously in rats (Zisch et al. 2003a). Similarly, bone regeneration was demonstrated in a critical size defect by cell-mediated proteolytic release of BMP from a matrix (Lutolf et al. 2003b). A further more natural mechanism of the control of growth factor binding, modulation and release is the attachment

of glycosaminoglycans to a material surface. These complex molecules have a tissue specific distribution and multiple physiological functions (Raman et al. 2005). Their sulphation patterns determine the specific interaction with proteins. One example is the binding of bFGF to heparin. Heparin has been widely incorporated into scaffolds to bind and release bFGF (Sakiyama-Elbert and Hubbell 2000; Zhang et al. 2006).

As demonstrated recently, the physiological effect of growth factors can be mimicked by designing of a modular peptide (Lee and Murphy 2010). This peptide contained a BMP-2 derived peptide sequence and hydroxyapatite-binding sequences inspired by the N-terminal alpha-helix of osteocalcin. The multifunctional fusion protein can bind to hydroxapatite coated surfaces or bone structures and exert BMP activity. When this peptide was presented to mesenchymal stem cells, both immobililized or in solution, the construct was capable to promote the osteogenic differentiation of the cells (Lee and Murphy 2010).

Microspheres with encapsulated or surface bound growth factors present a system to persist and deliver growth factors at the target site (Arras et al. 1998; Cleland et al. 2001; Park et al. 2009). For the fabrication of biodegradable polymer microspheres polyester like polylactide (PLA) and poly(lactic-co-glycolic acid) have been used. Applying a double emulsion technique, growth factors, such as bFGF, VEGF have been mixed into the particles (Perets et al. 2003). The loaded microspheres were incorporated into an alginate matrix or hydrogel. This approach enables the delivery of two or more growth factors with distinct kinetics. Microspheres containing PDGF were mixed with VEFG prior to processing into scaffolds, which resulted in a rapid release of VEGF and a slower, more even distribution of PDGF. When the scaffolds were implanted into rats, the distinct release kinetics of the growth factors stimulated the formation of a mature vasculature (Richardson et al. 2001).

24.3.2 Physical Modification to Control the Biointerface

24.3.2.1 Structural Organization of the Surface

The structure of a material surface can be categorized into topography and chemical patterning. The topography reflects the roughness of a surface which can be designed by ridges and grooves or by evenly or randomly distributed pits or protrusions. Chemical patterning is achieved by the spatial organization and immobilization of molecules in controllably size and position, mostly to control cell adhesion (Lim and Donahue 2007).

For clinical application of titanium implants different techniques have been used to roughen the surface, which include blasting, etching, and oxidation. A huge number of experimental data demonstrate that a rough implant surface has a beneficial effect on the bone response (Wennerberg and Albrektsson 2009). This concerns roughness in the micrometre level, whereas little is known about the effects of topographies in the nanometre level in vivo (Wennerberg and Albrektsson 2009). When testing the

cell behaviour on topographies the scale plays an important role. It became obvious that cells are able to sense the micro- and nanoscale topography and react with bridging of grooves or conforming the surface structure (Millette et al. 1987; Teixeira et al. 2003; Walboomers et al. 1999). The behaviour of the whole cell due to a topography was correlated with an orientation of the cytoskeleton and the alignment of focal adhesions (Dalby et al. 2002, 2003). In addition to structural changes in the organization of cellular components, functional consequences have been observed. Osteoblastic cells expressed a higher RNA level of osteopontin and osteocalcin when cultured on a surface with grooves than on a flat surface (Matsuzaka et al. 2004). Apparently, a defined size of pits or grooves is important on a structured surface. As shown, osteoblastic differentiation measured by the activity of alkaline phosphatase was stimulated more on 11 nm islands than on 85 nm islands (Lim et al. 2005). Similarly, also cell proliferation depends on defined surface structures. Progenitor cells displayed a higher proliferation rate on 5–40 μm diameter posts compared with cells on a smooth surface (Mata et al. 2002). In addition to the size of posts created on a surface the organization of a pattern controls the function of cells. When mesenchymal stem cells were cultured on disordered dots with nanosize the cells were induced to express osteocalcin and osteopontin in the absence of osteogenic supplements, demonstrating the stimulation of osteogenic differentiation (Dalby et al. 2007). In comparison, when the same nanofeatures were symmetrically organized, the cells did not express osteogenic proteins.

Although experiments are rare which demonstrate that a defined topography, regarding topographic size, shape or uniformity control a specific function of stem cells, it is obvious that micro- and nanostructured surfaces stimulate various collective cell functions (Lim and Donahue 2007).

Chemical patterning which generates precisely defined micro- or nanometer-areas for cell adhesion can be achieved by lithographic techniques (Nie and Kumacheva 2008). These techniques involve photolithography and printing techniques. Printing methods can be classified into techniques which involve the contact of a stamp with the substrate and methods which directly transfer “ink” to the substrate. Dip-pen nanolithography represents a relatively new direct writing technique, using the tip of an atomic force microscope to form a liquid meniscus between tip and substrate, and as a result of this procedure the ink molecules are transferred to the underlying substrate by chemical or physical adsorption (Piner et al. 1999). Micropatterning allows the spatial control of adhesion of the whole cell. By restriction of cell spreading the shape of cells can be controlled. Using mesenchymal stem cells, it was demonstrated that cell shape commits the direction of differentiation (McBeath et al. 2004). More rounded cells differentiated to adipocytes, whereas flat cells became osteocytes. The authors revealed that induction of mechanical tension of the cytoskeleton, which correlates with stress fibre formation and is mediated by the activities of RhoA and Rho kinase (ROCK) induces osteogenic differentiation. Blocking of RhoA and ROCK activities stimulated the adipogenic differentiation. By generating fibronectin lines in the nanoscale which altered the cell morphology, the proliferation of embryonic stem cells was stimulated, which depended on an altered organization of the cytoskeleton (Gerecht et al. 2007).

In addition to control of the entire cell shape by adhesion patterns, the sensing of nanoscale adhesion sites by cells controls integrin mediated signal transduction and in consequence influences differentiation and proliferation. For example, the precise spacing between nanotopographic features of RGD-peptides for cell adhesion can modulate the clustering of integrins. A minimal distance of 58 nm between adhesive dots was required for integrin clustering, formation of stable focal adhesions and cell spreading (Arnold et al. 2004; Cavalcanti-Adam et al. 2007). The formation of a molecular gradient of the ligand spacing from 50 to 80 nm revealed that cells are able to sense the small differences in ligand spacing (Arnold et al. 2008). Differences which are little as 1 nm seem to affect cell polarization and migration.

24.3.2.2 Mechanical Characteristics of the Surface

Mechanical stimuli represent regulators of development and function in many tissues. It is generally accepted that the structure of the various tissues reflect the acting forces, which specifically control the physiological processes. In some cases, tissues are heterogeneously organized into mechanically distinct zones, for example the superficial, radial and tight zones of cartilage. Therefore, implant materials must provide some level of physical support to assist tissue function. Engineering strategies have been developed to steer the viscoelastic properties of implant materials, for example by cross-linking of polymers. Highly elastic gels of cross-linked hyaluronic acid with controllable viscoelasticity were generated for tissue engineering of vocal folds (Sahiner et al. 2008). For tendon repair, gels were combined with a type I collagen sponge to optimize the stiffness of the material, which was successfully applied in a patellar tendon model (Butler et al. 2008). Findings in several cell types provide evidence for the importance of the substrate stiffness as a physical signal for cells (Georges and Janmey 2005). Early experiments demonstrated that differentiation of mammary epithelial cells increased when grown on soft collagen gel substrate, as opposed to tissue culture plastic (Emerman et al. 1979). Neurons preferentially branched on soft tissues compared to stiff surfaces (Flanagan et al. 2002). Although in most of these studies, the influence of different mechanical properties is difficult to separate from the type and density of the chemical ligand, it is obvious that stiffness of the substrate plays a role in tissue development. The role of substrate stiffness in the context with regenerative processes was emphasised by the fundamental finding that stem cell lineage specification can be determined by mechanical properties of the substrate (Engler et al. 2006). Mesenchymal stem cells were grown on polyacrylamide gels with varying compliance. These experiments convincingly demonstrated that the stiffness of the material defines the differentiation lineage (Discher et al. 2009; Zajac and Discher 2008). Soft substrates which mimic the mechanical properties of brain stimulated the neurogenic differentiation, intermediate stiffness leads to muscle cell differentiation and stiff substrates where found to be osteogenic. Similar experiments using adult neural stem cells have shown that softer substrates provoked neuronal differentiation, whereas stiffer materials induced

the formation of glial cells (Saha et al. 2008). The mechanical properties of the substrates were also found to control the self-renewal of stem cells. Adult stem cells from skeletal muscle tissue revealed increased cell proliferation with rising stiffness of the matrix (Boonen et al. 2009). Mesenchymal stem cells were kept quiescent on a gel that mimicked the softness of bone marrow. In contrast stiffer substrates induced the entry of these cells into the cell cycle (Winer et al. 2009). The cells maintained the multilineage potential and could be differentiated both to adipocytes and osteocytes. These experiments provided evidence of mimicking the functional capacity of a bone marrow niche by tuning the mechanical properties of an artificial substrate. In addition to the control of proliferation and multipotential differentiation, sensing of substrate stiffnesses enables cells to migrate from soft to stiffer matrices, which appears of importance for stem cell translocation to sites of tissue regeneration (Gray et al. 2003; Kidoaki and Matsuda 2008). This phenomenon was termed "durotaxis" (Lo et al. 2000).

24.4 Applications for Therapeutic Devices

Progress in biomaterials design and engineering are converging to enable a new generation of instructive materials to emerge as candidates for regenerative medicine. The aim of the design of current biomaterials is to regulate tissue regeneration by modulating direct or indirect chemical and physical control over transplanted or host cells. The dilemma is that to influence cell behaviour, biomaterials must provide complex information (Place et al. 2009). Tissue engineered skin equivalents have been introduced into clinical practice in 1997. Since then tissue engineered devices have been in clinical trials or already approved as therapies for tissues including cartilage, bone, blood vessel and pancreas. However, over-engineered devices make their translation to clinical use unlikely. The reconstruction of entire organs has largely given up and changed to smaller goals. For example, clinical advance in cardiac repair focus on coronar arteries, valves and regeneration of the myocardium. In principle, the aim is to develop synthetic materials that establish key interaction with cells that stimulate the innate organization and self-repair of the body.

24.5 Barriers to Practice and Prospects

A major hurdle for the progress in the application of biomaterials in the field of regenerative medicine lies not in the biomaterials but in stem-cell biology. The advancement of basic research in stem cell biology represents the driving factor for the development of biomaterials to regenerate a specific tissue. Current trends suggest that biomaterial development will continue to create more life-like multifunctional materials that are able to simultaneously provide complex biological signals (Chan and Mooney 2008; Howard et al. 2008). Much can be learned from

the mechanisms that regulate cell fate in the stem cell niche. For example, the adhesion molecules that contribute to asymmetric stem cell division have begun to identified within the niche environment of hair follicle, intestinal epithelial, spermatogonial stem cells (Kanatsu-Shinohara et al. 2008; Ohyama et al. 2006; Tanentzapf et al. 2007). In addition to the general control of stem cell function, there is growing interest in the dynamic nature of stem cell niches which can change properties under certain conditions (Adams and Scadden 2008).

24.6 Conclusions and Future Challenges

Chemical and physical characteristics of biomaterials are able to control the biology of stem cells and significant advances have been gained in in vitro studies. By controlling the properties of biomaterials we may further improve the regulation of stem cell in a bioartificial system. Although stem cell function is regulated by a set of different signals from the environment, the control of the extracellular matrix has proven a valuable tool to guide the development and commitment of stem cells. The challenge is to engineer an artificial extracellular matrix, which is capable to directly control the behaviour of stem cells. In addition, the outcome of growth factors administration can be improved enormously with the use of slow-release constructs. A further step in the generation of bioactive materials will be the design of heterogeneous constructs and even complex organs, which will require both more insights the mechanisms of cell and developmental biology as well as innovation in biomaterial research.

References

Adams GB, Scadden DT (2008) A niche opportunity for stem cell therapeutics. Gene Ther 15:96–99

Anderson DG, Levenberg S, Langer R (2004) Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. Nat Biotechnol 22:863–866

Antia M, Baneyx G, Kubow KE et al (2008) Fibronectin in aging extracellular matrix fibrils is progressively unfolded by cells and elicits an enhanced rigidity response. Faraday Discuss 139:229–249, discussion 309-25, 419-20

Arnold M, Cavalcanti-Adam EA, Glass R et al (2004) Activation of integrin function by nanopatterned adhesive interfaces. Chemphyschem 5:383–388

Arnold M, Hirschfeld-Warneken VC, Lohmuller T et al (2008) Induction of cell polarization and migration by a gradient of nanoscale variations in adhesive ligand spacing. Nano Lett 8:2063–2069

Arras M, Mollnau H, Strasser R et al (1998) The delivery of angiogenic factors to the heart by microsphere therapy. Nat Biotechnol 16:159–162

Augst AD, Kong HJ, Mooney DJ (2006) Alginate hydrogels as biomaterials. Macromol Biosci 6:623–633

Banerjee P, Irvine DJ, Mayes AM et al (2000) Polymer latexes for cell-resistant and cell-interactive surfaces. J Biomed Mater Res 50:331–339

Barrere F, van der Valk CM, Meijer G et al (2003) Osteointegration of biomimetic apatite coating applied onto dense and porous metal implants in femurs of goats. J Biomed Mater Res B Appl Biomater 67:655–665

Benoit DS, Anseth KS (2005) The effect on osteoblast function of colocalized RGD and PHSRN epitopes on PEG surfaces. Biomaterials 26:5209–5220

Bentz H, Schroeder JA, Estridge TD (1998) Improved local delivery of TGF-beta2 by binding to injectable fibrillar collagen via difunctional polyethylene glycol. J Biomed Mater Res 39:539–548

Blom EJ, Klein-Nulend J, Wolke JG et al (2002) Transforming growth factor-beta1 incorporation in an alpha-tricalcium phosphate/dicalcium phosphate dihydrate/tetracalcium phosphate monoxide cement: release characteristics and physicochemical properties. Biomaterials 23:1261–1268

Boonen KJ, Rosaria-Chak KY, Baaijens FP et al (2009) Essential environmental cues from the satellite cell niche: optimizing proliferation and differentiation. Am J Physiol Cell Physiol 296:C1338–C1345

Bulpitt P, Aeschlimann D (1999) New strategy for chemical modification of hyaluronic acid: preparation of functionalized derivatives and their use in the formation of novel biocompatible hydrogels. J Biomed Mater Res 47:152–169

Butler DL, Juncosa-Melvin N, Boivin GP et al (2008) Functional tissue engineering for tendon repair: a multidisciplinary strategy using mesenchymal stem cells, bioscaffolds, and mechanical stimulation. J Orthop Res 26:1–9

Carson AE, Barker TH (2009) Emerging concepts in engineering extracellular matrix variants for directing cell phenotype. Regen Med 4:593–600

Cartmell S (2009) Controlled release scaffolds for bone tissue engineering. J Pharm Sci 98:430–441

Cavalcanti-Adam EA, Volberg T, Micoulet A et al (2007) Cell spreading and focal adhesion dynamics are regulated by spacing of integrin ligands. Biophys J 92:2964–2974

Chan G, Mooney DJ (2008) New materials for tissue engineering: towards greater control over the biological response. Trends Biotechnol 26:382–392

Choi CK, Vicente-Manzanares M, Zareno J et al (2008) Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. Nat Cell Biol 10:1039–1050

Cleland JL, Duenas ET, Park A et al (2001) Development of poly-(D, L-lactide–coglycolide) microsphere formulations containing recombinant human vascular endothelial growth factor to promote local angiogenesis. J Control Release 72:13–24

Cluzel C, Saltel F, Lussi J et al (2005) The mechanisms and dynamics of (alpha)v(beta)3 integrin clustering in living cells. J Cell Biol 171:383–392

Cohen M, Kam Z, Addadi L et al (2006) Dynamic study of the transition from hyaluronan- to integrin-mediated adhesion in chondrocytes. EMBO J 25:302–311

Comisar WA, Kazmers NH, Mooney DJ et al (2007) Engineering RGD nanopatterned hydrogels to control preosteoblast behavior: a combined computational and experimental approach. Biomaterials 28:4409–4417

Cordonnier T, Layrolle P, Gaillard J et al (2010) 3D environment on human mesenchymal stem cells differentiation for bone tissue engineering. J Mater Sci Mater Med 21:981–987

Cox BD, Natarajan M, Stettner MR et al (2006) New concepts regarding focal adhesion kinase promotion of cell migration and proliferation. J Cell Biochem 99:35–52

Craig WS, Cheng S, Mullen DG et al (1995) Concept and progress in the development of RGD-containing peptide pharmaceuticals. Biopolymers 37:157–175

Crisan M, Yap S, Casteilla L et al (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3:301–313

Crisp M, Liu Q, Roux K et al (2006) Coupling of the nucleus and cytoplasm: role of the LINC complex. J Cell Biol 172:41–53

Curran JM, Chen R, Hunt JA (2005) Controlling the phenotype and function of mesenchymal stem cells in vitro by adhesion to silane-modified clean glass surfaces. Biomaterials 26:7057–7067

Curran JM, Chen R, Hunt JA (2006) The guidance of human mesenchymal stem cell differentiation in vitro by controlled modifications to the cell substrate. Biomaterials 27:4783–4793
Dalby MJ, Yarwood SJ, Riehle MO et al (2002) Increasing fibroblast response to materials using nanotopography: morphological and genetic measurements of cell response to 13-nm-high polymer demixed islands. Exp Cell Res 276:1–9
Dalby MJ, Childs S, Riehle MO et al (2003) Fibroblast reaction to island topography: changes in cytoskeleton and morphology with time. Biomaterials 24:927–935
Dalby MJ, Gadegaard N, Tare R et al (2007) The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. Nat Mater 6:997–1003
Daley WP, Peters SB, Larsen M (2008) Extracellular matrix dynamics in development and regenerative medicine. J Cell Sci 121:255–264
de Groot K, Wolke JG, Jansen JA (1998) Calcium phosphate coatings for medical implants. Proc Inst Mech Eng H 212:137–147
de Jonge LT, Leeuwenburgh SC, Wolke JG et al (2008) Organic-inorganic surface modifications for titanium implant surfaces. Pharm Res 25:2357–2369
Dechat T, Pfleghaar K, Sengupta K et al (2008) Nuclear lamins: major factors in the structural organization and function of the nucleus and chromatin. Genes Dev 22:832–853
del Rio A, Perez-Jimenez R, Liu R et al (2009) Stretching single talin rod molecules activates vinculin binding. Science 323:638–641
DeLong SA, Moon JJ, West JL (2005) Covalently immobilized gradients of bFGF on hydrogel scaffolds for directed cell migration. Biomaterials 26:3227–3234
Discher DE, Mooney DJ, Zandstra PW (2009) Growth factors, matrices, and forces combine and control stem cells. Science 324:1673–1677
Drumheller PD, Hubbell JA (1995) Densely crosslinked polymer networks of poly(ethylene glycol) in trimethylolpropane triacrylate for cell-adhesion-resistant surfaces. J Biomed Mater Res 29:207–215
Durrieu MC, Pallu S, Guillemot F et al (2004) Grafting RGD containing peptides onto hydroxyapatite to promote osteoblastic cells adhesion. J Mater Sci Mater Med 15:779–786
Ehrlicher AJ, Nakamura F, Hartwig H et al (2011) Mechanical strain in actin networks regulates FilGAP and integrin binding to filamin A. Nature 478:260–263
El-Ghannam A (2005) Bone reconstruction: from bioceramics to tissue engineering. Expert Rev Med Devices 2:87–101
Emerman JT, Burwen SJ, Pitelka DR (1979) Substrate properties influencing ultrastructural differentiation of mammary epithelial cells in culture. Tissue Cell 11:109–119
Engler AJ, Sen S, Sweeney HL et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126:677–689
Evanko SP, Tammi MI, Tammi RH et al (2007) Hyaluronan-dependent pericellular matrix. Adv Drug Deliv Rev 59:1351–1365
Feldherr CM, Akin D (1990) The permeability of the nuclear envelope in dividing and nondividing cell cultures. J Cell Biol 111:1–8
Finke B, Luethen F, Schroeder K et al (2007) The effect of positively charged plasma polymerization on initial osteoblastic focal adhesion on titanium surfaces. Biomaterials 28:4521–4534
Flanagan LA, Ju YE, Marg B et al (2002) Neurite branching on deformable substrates. Neuroreport 13:2411–2415
Flanagan LA, Rebaza LM, Derzic S et al (2006) Regulation of human neural precursor cells by laminin and integrins. J Neurosci Res 83:845–856
Fujisawa R, Mizuno M, Nodasaka Y et al (1997) Attachment of osteoblastic cells to hydroxyapatite crystals by a synthetic peptide (Glu7-Pro-Arg-Gly-Asp-Thr) containing two functional sequences of bone sialoprotein. Matrix Biol 16:21–28
Galbraith CG, Yamada KM, Galbraith JA (2007) Polymerizing actin fibers position integrins primed to probe for adhesion sites. Science 315:992–995
Gallant ND, Michael KE, Garcia AJ (2005) Cell adhesion strengthening: contributions of adhesive area, integrin binding, and focal adhesion assembly. Mol Biol Cell 16:4329–4340

Geiger B, Bershadsky A, Pankov R et al (2001) Transmembrane crosstalk between the extracellular matrix–cytoskeleton crosstalk. Nat Rev Mol Cell Biol 2:793–805
Geiger B, Spatz JP, Bershadsky AD (2009) Environmental sensing through focal adhesions. Nat Rev Mol Cell Biol 10:21–33
Georges PC, Janmey PA (2005) Cell type-specific response to growth on soft materials. J Appl Physiol 98:1547–1553
Gerecht S, Bettinger CJ, Zhang Z et al (2007) The effect of actin disrupting agents on contact guidance of human embryonic stem cells. Biomaterials 28:4068–4077
Giannone G, Dubin-Thaler BJ, Rossier O et al (2007) Lamellipodial actin mechanically links myosin activity with adhesion-site formation. Cell 128:561–575
Gilbert M, Shaw WJ, Long JR et al (2000) Chimeric peptides of statherin and osteopontin that bind hydroxyapatite and mediate cell adhesion. J Biol Chem 275:16213–16218
Girotti A, Reguera J, Rodriguez-Cabello JC et al (2004) Design and bioproduction of a recombinant multi(bio)functional elastin-like protein polymer containing cell adhesion sequences for tissue engineering purposes. J Mater Sci Mater Med 15:479–484
Godfrey EW, Gradall KS (1998) Basal lamina molecules are concentrated in myogenic regions of the mouse limb bud. Anat Embryol (Berl) 198:481–486
Gray DS, Tien J, Chen CS (2003) Repositioning of cells by mechanotaxis on surfaces with micropatterned Young's modulus. J Biomed Mater Res A 66:605–614
Groll J, Fiedler J, Engelhard E et al (2005) A novel star PEG-derived surface coating for specific cell adhesion. J Biomed Mater Res A 74:607–617
Habraken WJ, Wolke JG, Jansen JA (2007) Ceramic composites as matrices and scaffolds for drug delivery in tissue engineering. Adv Drug Deliv Rev 59:234–248
Haque F, Lloyd DJ, Smallwood DT et al (2006) SUN1 interacts with nuclear lamin A and cytoplasmic nesprins to provide a physical connection between the nuclear lamina and the cytoskeleton. Mol Cell Biol 26:3738–3751
Hersel U, Dahmen C, Kessler H (2003) RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials 24:4385–4415
Hiemstra C, Zhong Z, van Steenbergen MJ et al (2007) Release of model proteins and basic fibroblast growth factor from in situ forming degradable dextran hydrogels. J Control Release 122:71–78
Hosseinkhani H, Hosseinkhani M, Tian F et al (2006) Osteogenic differentiation of mesenchymal stem cells in self-assembled peptide-amphiphile nanofibers. Biomaterials 27:4079–4086
Howard D, Buttery LD, Shakesheff KM et al (2008) Tissue engineering: strategies, stem cells and scaffolds. J Anat 213:66–72
Humphries JD, Wang P, Streuli C et al (2007) Vinculin controls focal adhesion formation by direct interactions with talin and actin. J Cell Biol 179:1043–1057
Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. Cell 110:673–687
Hynes RO (2009) The extracellular matrix: not just pretty fibrils. Science 326:1216–1219
Ingber DE (1997) Tensegrity: the architectural basis of cellular mechanotransduction. Annu Rev Physiol 59:575–599
Ingber DE (2006) Mechanical control of tissue morphogenesis during embryological development. Int J Dev Biol 50:255–266
Itoh D, Yoneda S, Kuroda S et al (2002) Enhancement of osteogenesis on hydroxyapatite surface coated with synthetic peptide (EEEEEEEPRGDT) in vitro. J Biomed Mater Res 62:292–298
Jansen JA, Vehof JW, Ruhe PQ et al (2005) Growth factor-loaded scaffolds for bone engineering. J Control Release 101:127–136
Jensen UB, Lowell S, Watt FM (1999) The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis. Development 126:2409–2418
Jo S, Engel PS, Mikos AG (2000) Synthesis of poly(ehtylene glycol)-tethered poly(propylene fumarate) and its modification with GRGD peptide. Polymer 41:7595–7604
Jones DL, Wagers AJ (2008) No place like home: anatomy and function of the stem cell niche. Nat Rev Mol Cell Biol 9:11–21

Kanatsu-Shinohara M, Takehashi M, Takashima S et al (2008) Homing of mouse spermatogonial stem cells to germline niche depends on beta1-integrin. Cell Stem Cell 3:533–542
Kanematsu A, Yamamoto S, Ozeki M et al (2004) Collagenous matrices as release carriers of exogenous growth factors. Biomaterials 25:4513–4520
Kantlehner M, Schaffner P, Finsinger D et al (2000) Surface coating with cyclic RGD peptides stimulates osteoblast adhesion and proliferation as well as bone formation. Chembiochem 1:107–114
Katoh K, Kano Y, Amano M et al (2001) Rho-kinase-mediated contraction of isolated stress fibers. J Cell Biol 153:569–584
Keselowsky BG, Collard DM, Garcia AJ (2003) Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. J Biomed Mater Res A 66:247–259
Keselowsky BG, Collard DM, Garcia AJ (2004) Surface chemistry modulates focal adhesion composition and signaling through changes in integrin binding. Biomaterials 25:5947–5954
Keselowsky BG, Collard DM, Garcia AJ (2005) Integrin binding specificity regulates biomaterial surface chemistry effects on cell differentiation. Proc Natl Acad Sci USA 102:5953–5957
Kidoaki S, Matsuda T (2008) Microelastic gradient gelatinous gels to induce cellular mechanotaxis. J Biotechnol 133:225–230
Kilpadi KL, Chang PL, Bellis SL (2001) Hydroxylapatite binds more serum proteins, purified integrins, and osteoblast precursor cells than titanium or steel. J Biomed Mater Res 57:258–267
Kim M, Carman CV, Yang W et al (2004) The primacy of affinity over clustering in regulation of adhesiveness of the integrin {alpha}L{beta}2. J Cell Biol 167:1241–1253
Klees RF, Salasznyk RM, Kingsley K et al (2005) Laminin-5 induces osteogenic gene expression in human mesenchymal stem cells through an ERK-dependent pathway. Mol Biol Cell 16:881–890
Klotzsch E, Smith ML, Kubow KE et al (2009) Fibronectin forms the most extensible biological fibers displaying switchable force-exposed cryptic binding sites. Proc Natl Acad Sci USA 106:18267–18272
Kolf CM, Cho E, Tuan RS (2007) Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Res Ther 9:204
Kroese-Deutman HC, Ruhe PQ, Spauwen PH et al (2005) Bone inductive properties of rhBMP-2 loaded porous calcium phosphate cement implants inserted at an ectopic site in rabbits. Biomaterials 26:1131–1138
Kuhl PR, Griffith-Cima LG (1996) Tethered epidermal growth factor as a paradigm for growth factor-induced stimulation from the solid phase. Nat Med 2:1022–1027
Kumar S, Maxwell IZ, Heisterkamp A et al (2006) Viscoelastic retraction of single living stress fibers and its impact on cell shape, cytoskeletal organization, and extracellular matrix mechanics. Biophys J 90:3762–3773
Kundu AK, Putnam AJ (2006) Vitronectin and collagen I differentially regulate osteogenesis in mesenchymal stem cells. Biochem Biophys Res Commun 347:347–357
Lee KY, Mooney DJ (2001) Hydrogels for tissue engineering. Chem Rev 101:1869–1879
Lee JS, Murphy WL (2010) Modular peptides promote human mesenchymal stem cell differentiation on biomaterial surfaces. Acta Biomater 6:21–28
Lee SH, Shin H (2007) Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering. Adv Drug Deliv Rev 59:339–359
Lee KY, Peters MC, Anderson KW et al (2000) Controlled growth factor release from synthetic extracellular matrices. Nature 408:998–1000
Leeuwenburgh SC, Wolke JG, Siebers MC et al (2006) In vitro and in vivo reactivity of porous, electrosprayed calcium phosphate coatings. Biomaterials 27:3368–3378
Legate KR, Wickstrom SA, Fassler R (2009) Genetic and cell biological analysis of integrin outside-in signaling. Genes Dev 23:397–418
Lim JY, Donahue HJ (2007) Cell sensing and response to micro- and nanostructured surfaces produced by chemical and topographic patterning. Tissue Eng 13:1879–1891

Lim JY, Hansen JC, Siedlecki CA et al (2005) Osteoblast adhesion on poly(L-lactic acid)/polystyrene demixed thin film blends: effect of nanotopography, surface chemistry, and wettability. Biomacromolecules 6:3319–3327
Lin HB, Sun W, Mosher DF et al (1994) Synthesis, surface, and cell-adhesion properties of polyurethanes containing covalently grafted RGD-peptides. J Biomed Mater Res 28:329–342
Lo CM, Wang HB, Dembo M et al (2000) Cell movement is guided by the rigidity of the substrate. Biophys J 79:144–152
Lozito TP, Kuo CK, Taboas JM et al (2009) Human mesenchymal stem cells express vascular cell phenotypes upon interaction with endothelial cell matrix. J Cell Biochem 107:714–722
Luo BH, Carman CV, Springer TA (2007) Structural basis of integrin regulation and signaling. Annu Rev Immunol 25:619–647
Lutolf MP, Lauer-Fields JL, Schmoekel HG et al (2003a) Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: engineering cell-invasion characteristics. Proc Natl Acad Sci USA 100:5413–5418
Lutolf MP, Weber FE, Schmoekel HG et al (2003b) Repair of bone defects using synthetic mimetics of collagenous extracellular matrices. Nat Biotechnol 21:513–518
Maeda T, Titani K, Sekiguchi K (1994) Cell-adhesive activity and receptor-binding specificity of the laminin-derived YIGSR sequence grafted onto Staphylococcal protein A. J Biochem 115:182–189
Mammoto A, Ingber DE (2009) Cytoskeletal control of growth and cell fate switching. Curr Opin Cell Biol 21:864–870
Maniotis AJ, Chen CS, Ingber DE (1997) Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. Proc Natl Acad Sci USA 94:849–854
Mann BK, Schmedlen RH, West JL (2001) Tethered-TGF-beta increases extracellular matrix production of vascular smooth muscle cells. Biomaterials 22:439–444
Mata A, Boehm C, Fleischman AJ et al (2002) Growth of connective tissue progenitor cells on microtextured polydimethylsiloxane surfaces. J Biomed Mater Res 62:499–506
Matsuzaka K, Yoshinari M, Shimono M et al (2004) Effects of multigrooved surfaces on osteoblast-like cells in vitro: scanning electron microscopic observation and mRNA expression of osteopontin and osteocalcin. J Biomed Mater Res A 68:227–234
McBeath R, Pirone DM, Nelson CM et al (2004) Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell 6:483–495
Mei YGS, Taylor M, Urquhart AJ, Bogatyrev SR, Cho SW, Davies MC, Alexander MR, Langer RS, Anderson DG (2009) Mapping the interaction among biomaterials, adsorbed proteins, and human embryonic stem cells. Adv Mater 21:2781–2786
Michael KE, Dumbauld DW, Burns KL et al (2009) Focal adhesion kinase modulates cell adhesion strengthening via integrin activation. Mol Biol Cell 20:2508–2519
Millette JR, Clark PJ, Boone RL et al (1987) Occurrence and biological activity testing of particulates in drinking water. Bull Environ Contam Toxicol 38:1–8
Miyamoto S, Teramoto H, Gutkind JS et al (1996) Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. J Cell Biol 135:1633–1642
Moreau JL, Xu HH (2009) Mesenchymal stem cell proliferation and differentiation on an injectable calcium phosphate-chitosan composite scaffold. Biomaterials 30:2675–2682
Moro L, Venturino M, Bozzo C et al (1998) Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. EMBO J 17:6622–6632
Muller P, Bulnheim U, Diener A et al (2008) Calcium phosphate surfaces promote osteogenic differentiation of mesenchymal stem cells. J Cell Mol Med 12:281–291
Nebe B, Finke B, Luthen F et al (2007) Improved initial osteoblast functions on amino-functionalized titanium surfaces. Biomol Eng 24:447–454
Nie Z, Kumacheva E (2008) Patterning surfaces with functional polymers. Nat Mater 7:277–290
Nilsson SK, Johnston HM, Whitty GA et al (2005) Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood 106:1232–1239

Ohyama M, Terunuma A, Tock CL et al (2006) Characterization and isolation of stem cell-enriched human hair follicle bulge cells. J Clin Invest 116:249–260
Page-McCaw A, Ewald AJ, Werb Z (2007) Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 8:221–233
Park YD, Tirelli N, Hubbell JA (2003) Photopolymerized hyaluronic acid-based hydrogels and interpenetrating networks. Biomaterials 24:893–900
Park JS, Na K, Woo DG et al (2009) Determination of dual delivery for stem cell differentiation using dexamethasone and TGF-beta3 in/on polymeric microspheres. Biomaterials 30:4796–4805
Perets A, Baruch Y, Weisbuch F et al (2003) Enhancing the vascularization of three-dimensional porous alginate scaffolds by incorporating controlled release basic fibroblast growth factor microspheres. J Biomed Mater Res A 65:489–497
Peterson LJ, Rajfur Z, Maddox AS et al (2004) Simultaneous stretching and contraction of stress fibers in vivo. Mol Biol Cell 15:3497–3508
Petrie TA, Raynor JE, Reyes CD et al (2008) The effect of integrin-specific bioactive coatings on tissue healing and implant osseointegration. Biomaterials 29:2849–2857
Phillips JE, Petrie TA, Creighton FP et al (2010) Human mesenchymal stem cell differentiation on self-assembled monolayers presenting different surface chemistries. Acta Biomater 6:12–20
Piner RD, Zhu J, Xu F et al (1999) "Dip-Pen" nanolithography. Science 283:661–663
Place ES, Evans ND, Stevens MM (2009) Complexity in biomaterials for tissue engineering. Nat Mater 8:457–470
Pommerenke H, Schmidt C, Durr F et al (2002) The mode of mechanical integrin stressing controls intracellular signaling in osteoblasts. J Bone Miner Res 17:603–611
Puklin-Faucher E, Sheetz MP (2009) The mechanical integrin cycle. J Cell Sci 122:179–186
Puklin-Faucher E, Gao M, Schulten K et al (2006) How the headpiece hinge angle is opened: new insights into the dynamics of integrin activation. J Cell Biol 175:349–360
Raman R, Sasisekharan V, Sasisekharan R (2005) Structural insights into biological roles of protein-glycosaminoglycan interactions. Chem Biol 12:267–277
Ratner BD (1995) Surface modification of polymers: chemical, biological and surface analytical challenges. Biosens Bioelectron 10:797–804
Rezania AJR, Lefkow AR, Healy KE (1999) Bioactivation of metal oxide surfaces. Langmuir 15:6931–6939
Richardson TP, Peters MC, Ennett AB et al (2001) Polymeric system for dual growth factor delivery. Nat Biotechnol 19:1029–1034
Ridley H (1952) Intra-ocular acrylic lenses after cataract extraction. Lancet 1:118–121
Riveline D, Zamir E, Balaban NQ et al (2001) Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. J Cell Biol 153:1175–1186
Rozario T, Desimone DW (2010) The extracellular matrix in development and morphogenesis: a dynamic view. Dev Biol 341:126–140
Ruhe PQ, Kroese-Deutman HC, Wolke JG et al (2004) Bone inductive properties of rhBMP-2 loaded porous calcium phosphate cement implants in cranial defects in rabbits. Biomaterials 25:2123–2132
Ruhe PQ, Boerman OC, Russel FG et al (2006) In vivo release of rhBMP-2 loaded porous calcium phosphate cement pretreated with albumin. J Mater Sci Mater Med 17:919–927
Saha K, Keung AJ, Irwin EF et al (2008) Substrate modulus directs neural stem cell behavior. Biophys J 95:4426–4438
Sahiner N, Jha AK, Nguyen D et al (2008) Fabrication and characterization of cross-linkable hydrogel particles based on hyaluronic acid: potential application in vocal fold regeneration. J Biomater Sci Polym Ed 19:223–243
Sakiyama-Elbert SE, Hubbell JA (2000) Development of fibrin derivatives for controlled release of heparin-binding growth factors. J Control Release 65:389–402
Salasznyk RM, Klees RF, Hughlock MK et al (2004) ERK signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells on collagen I and vitronectin. Cell Commun Adhes 11:137–153

Schenke-Layland K, Angelis E, Rhodes KE et al (2007) Collagen IV induces trophoectoderm differentiation of mouse embryonic stem cells. Stem Cells 25:1529–1538
Schmidt C, Pommerenke H, Durr F et al (1998) Mechanical stressing of integrin receptors induces enhanced tyrosine phosphorylation of cytoskeletally anchored proteins. J Biol Chem 273: 5081–5085
Schneller M, Vuori K, Ruoslahti E (1997) Alphavbeta3 integrin associates with activated insulin and PDGFbeta receptors and potentiates the biological activity of PDGF. EMBO J 16:5600–5607
Schober M, Raghavan S, Nikolova M et al (2007) Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics. J Cell Biol 176:667–680
Seeherman H, Wozney JM (2005) Delivery of bone morphogenetic proteins for orthopedic tissue regeneration. Cytokine Growth Factor Rev 16:329–345
Silva GA, Czeisler C, Niece KL et al (2004) Selective differentiation of neural progenitor cells by high-epitope density nanofibers. Science 303:1352–1355
Silva AK, Richard C, Bessodes M et al (2009) Growth factor delivery approaches in hydrogels. Biomacromolecules 10:9–18
Sivakumar P, Czirok A, Rongish BJ et al (2006) New insights into extracellular matrix assembly and reorganization from dynamic imaging of extracellular matrix proteins in living osteoblasts. J Cell Sci 119:1350–1360
Soriano I, Evora C (2000) Formulation of calcium phosphates/poly (d, l-lactide) blends containing gentamicin for bone implantation. J Control Release 68:121–134
Stamenkovic I (2003) Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol 200:448–464
Stamenovic D, Fredberg JJ, Wang N et al (1996) A microstructural approach to cytoskeletal mechanics based on tensegrity. J Theor Biol 181:125–136
Sun H, Ye F, Wang J et al (2008) The upregulation of osteoblast marker genes in mesenchymal stem cells prove the osteoinductivity of hydroxyapatite/tricalcium phosphate biomaterial. Transplant Proc 40:2645–2648
Takada Y, Ye X, Simon S (2007) The integrins. Genome Biol 8:215
Tanentzapf G, Devenport D, Godt D et al (2007) Integrin-dependent anchoring of a stem-cell niche. Nat Cell Biol 9:1413–1418
Teixeira AI, Abrams GA, Bertics PJ et al (2003) Epithelial contact guidance on well-defined micro- and nanostructured substrates. J Cell Sci 116:1881–1892
van der Flier A, Sonnenberg A (2001) Function and interactions of integrins. Cell Tissue Res 305:285–298
Vogel V (2006) Mechanotransduction involving multimodular proteins: converting force into biochemical signals. Annu Rev Biophys Biomol Struct 35:459–488
Vogel V, Sheetz MP (2009) Cell fate regulation by coupling mechanical cycles to biochemical signaling pathways. Curr Opin Cell Biol 21:38–46
Walboomers XF, Monaghan W, Curtis AS et al (1999) Attachment of fibroblasts on smooth and microgrooved polystyrene. J Biomed Mater Res 46:212–220
Walschus U, Hoene A, Neumann HG et al (2009) Morphometric immunohistochemical examination of the inflammatory tissue reaction after implantation of calcium phosphate-coated titanium plates in rats. Acta Biomater 5:776–784
Wang N, Butler JP, Ingber DE (1993) Mechanotransduction across the cell surface and through the cytoskeleton. Science 260:1124–1127
Wang N, Tytell JD, Ingber DE (2009) Mechanotransduction at a distance: mechanically coupling the extracellular matrix with the nucleus. Nat Rev Mol Cell Biol 10:75–82
Wehrle-Haller B, Imhof B (2002) The inner lives of focal adhesions. Trends Cell Biol 12:382–389
Wehrle-Haller B, Imhof BA (2003) Integrin-dependent pathologies. J Pathol 200:481–487
Wennerberg A, Albrektsson T (2009) Effects of titanium surface topography on bone integration: a systematic review. Clin Oral Implants Res 20(Suppl 4):172–184
Wiesner S, Legate KR, Fassler R (2005) Integrin-actin interactions. Cell Mol Life Sci 62:1081–1099

Wijelath ES, Rahman S, Namekata M et al (2006) Heparin-II domain of fibronectin is a vascular endothelial growth factor-binding domain: enhancement of VEGF biological activity by a singular growth factor/matrix protein synergism. Circ Res 99:853–860

Winer JP, Janmey PA, McCormick ME et al (2009) Bone marrow-derived human mesenchymal stem cells become quiescent on soft substrates but remain responsive to chemical or mechanical stimuli. Tissue Eng Part A 15:147–154

Yoshida N, Hishiyama S, Yamaguchi M et al (2003) Decrease in expression of alpha 5 beta 1 integrin during neuronal differentiation of cortical progenitor cells. Exp Cell Res 287:262–271

Zaidel-Bar R, Ballestrem C, Kam Z et al (2003) Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. J Cell Sci 116:4605–4613

Zaidel-Bar R, Itzkovitz S, Ma'ayan A et al (2007) Functional atlas of the integrin adhesome. Nat Cell Biol 9:858–867

Zajac AL, Discher DE (2008) Cell differentiation through tissue elasticity-coupled, myosin-driven remodeling. Curr Opin Cell Biol 20:609–615

Zhang L, Furst EM, Kiick KL (2006) Manipulation of hydrogel assembly and growth factor delivery via the use of peptide-polysaccharide interactions. J Control Release 114:130–142

Zhang X, Jiang G, Cai Y et al (2008) Talin depletion reveals independence of initial cell spreading from integrin activation and traction. Nat Cell Biol 10:1062–1068

Zhao J, Guan JL (2009) Signal transduction by focal adhesion kinase in cancer. Cancer Metastasis Rev 28:35–49

Ziegler J, Mayr-Wohlfart U, Kessler S et al (2002) Adsorption and release properties of growth factors from biodegradable implants. J Biomed Mater Res 59:422–428

Zisch AH, Lutolf MP, Ehrbar M et al (2003a) Cell-demanded release of VEGF from synthetic, biointeractive cell ingrowth matrices for vascularized tissue growth. FASEB J 17:2260–2262

Zisch AH, Lutolf MP, Hubbell JA (2003b) Biopolymeric delivery matrices for angiogenic growth factors. Cardiovasc Pathol 12:295–310

Part IV
Regenerative Therapies

Chapter 25
Emerging Concepts in Myocardial Pharmacoregeneration

Laura C. Zelarayán, Maria Patapia Zafiriou, and Wolfram-Hubertus Zimmermann

Abstract Novel pharmacological approaches addressing the underlying problem of heart failure development, namely progressive cardiomyocyte loss, are emerging. The main therapeutic aims are to either protect cardiomyocytes from ischemia-associated stressors or to facilitate endogenous regeneration. The latter may be achieved by (1) induction of cardiomyocyte proliferation or (2) activation of recently identified dormant resident cardiac progenitor cell pools. The development of pharmacological approaches to enhance these under normal circumstances ineffective self-repair mechanisms would be highly exciting and set the stage for the new therapeutic concept of "myocardial pharmacoregeneration". This chapter will first delineate the phenotype of resident cardiac progenitor cells and then summarize growth factors/peptides as well as small molecules presently under investigation as candidates for pharmacoregeneration of the heart.

25.1 Introduction

The assumption that bone marrow contains progenitor or stem cell populations with cardio-regenerative potential (Orlic et al. 2001a, b) has prompted several stem cell-base clinical trials in the last years (Laflamme and Murry 2011). Most of these trials showed no or only modest benefits (Segers and Lee 2008). Although underlying mechanisms of the therapeutic effect of cell-based therapies observed in some studies remain speculative, evidence for paracrine mechanisms rather than functional integration of exogenous stem cells and derivatives is accumulating (Mirotsou et al. 2011). Deciphering the therapeutic potential of cell-mediated paracrine mechanisms

L.C. Zelarayán • M.P. Zafiriou • W.-H. Zimmermann (✉)
Department of Pharmacology, Heart Research Center Goettingen,
University Medical Center Goettingen,
Robert-Koch-Str. 40, Goettingen 37075, Germany
e-mail: w.zimmermann@med.uni-goettingen.de

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_25,

and enhancing them pharmacologically would be clinically extremely attractive; especially considering the insufficient available pharmacological treatment, which delays but does not prevent the onset of end-stage heart failure.

The investigation of the regenerative capacity of the adult mammalian heart has generated ambiguous results (Soonpaa and Field 1998; Anversa et al. 2007; Bergmann et al. 2009; Kajstura et al. 2010). Despite the contention in the field, there is agreement that endogenous myocardial regeneration is insufficient to stop disease progression into end-stage heart failure. Guideline-based drug therapy can reduce symptoms, shield the heart from neurohumoral overstimulation, and control pathological remodeling, but it fails to regenerate the heart. Insufficient myocardial regeneration in the mammalian heart is in clear contrast to the remarkable regeneration capacity in lower vertebrates, such as zebrafish and newts. Here little residual injury can be observed after removal of up to 20% of the heart (Poss et al. 2002; Lepilina et al. 2006). The phylogenetic distance between fish and men has apparently been a barrier for translating mechanistic insight from lower vertebrates into clinical therapy. However, recent observations that neonatal mice can regenerate their heart similarly as zebrafish (Porrello et al. 2011) have reactivated the search for signals that may also control myocardial regeneration in the adult mammalian heart. Clinical data from intrauterine surgery confirms that the human heart is essentially capable of regeneration (Herdrich et al. 2010). However, limited access to fetal and early postnatal human myocardium restricts studies aiming at the identification of mechanisms underlying human heart regeneration. Whether the observed regeneration is the consequence of cardiomyocyte proliferation or activation of cardiac progenitors and why it cannot be efficiently activated in the adult heart remains unknown.

Cardiac stem or progenitor cells (the term cardiac progenitor cells or CPCs will be used to describe this cell population along the whole chapter) have recently been identified in the mouse and human heart (Beltrami et al. 2003). CPCs may represent remnants of embryonic heart development, but their origin and postnatal relevance remain a matter of debate (Sussman and Murry 2008). When isolated from the heart, these cells appear to exhibit the potential to differentiate into most cellular components of the heart, including endothelial cells, smooth muscle cells, and cardiomyocytes. Using *in vivo* genetic lineage tracing, Hsieh et al. reported the generation of new cardiomyocytes after adult heart injury potentially from a progenitor cell pool (Hsieh et al. 2007). However, further studies are necessary to better define the elusive nature of resident CPCs in the heart and identify the reason for their insufficient functionality especially in the injured postnatal human heart. To define unique CPC properties, *in vitro* studies may be particularly useful as they may help to identify distinct paracrine factors secreted from CPCs or in response to CPC activity under controlled conditions (Amado et al. 2005; Urbich et al. 2005; Psaltis et al. 2010; Tang et al. 2010). This would help to delineate components of signaling pathways as potential drug targets for the induction of cardiomyocyte regeneration (Mirotsou et al. 2007; Zelarayan et al. 2008; Oerlemans et al. 2010; Oikonomopoulos et al. 2011), and to effectively screen small molecules for their capacity to regulate CPC activity (Sadek et al. 2008; Russell et al. 2012). Collectively, there are encouraging

data that provide a solid rationale for the further development of the concept of myocardial pharmacoregeneration (Kanashiro-Takeuchi et al. 2011).

Coming from the "historical" perspective of bone marrow cell activation as means to regenerate the heart, this chapter will (1) provide an overview of the biomarkers that define resident CPCs and (2) summarize available data on pharmacological activation of endogenous cellular repair mechanisms.

25.2 Cell-Based Heart Repair

25.2.1 Bone-Marrow Derived Cells

The bone marrow is a reservoir for hematopoietic (HSCs), mesenchymal (MSCs), and endothelial (EPCs) stem/progenitor cells. These progenitors can be readily isolated from the bone marrow's mononuclear cell (MNC) fraction (Fernandez-Aviles et al. 2004). MSCs appear particularly interesting given their documented multilineage potential with an *in vitro* and/or *in vivo* capacity to differentiate into osteoblasts, chondrocytes, myocytes, adipocytes, beta-pancreatic islets, and neuronal cells (Pittenger et al. 1999). Initial efforts to move animal experiments into the clinic using bone marrow-derived cells for cardiac repair were triggered by the observation of their regenerative capacity in small animal models (Orlic et al. 2001a, b; Yoon et al. 2005). However, several other studies failed to replicate these findings and showed that bone marrow-derived stem cells do not form cardiomyocytes, but instead adopt characteristic haematopoietic fates after transplantation in ischemic hearts (Balsam et al. 2004; Murry et al. 2004; Laflamme and Murry 2011). Nevertheless, multiple clinical trials have now provided hints that bone marrow-derived mononuclear cells have the capacity to improve myocardial performance post-myocardial infarction, likely via paracrine mechanisms (Abdel-Latif et al. 2007; Dawn et al. 2009; Wei et al. 2009). A large multicenter phase III trial, the Bone Marrow Therapy for Acute Myocardial Infarction (BAMI) trial (ClinicalTrials.gov Identifier: NCT01569178), will provide hopefully unambiguous insight into the clinical utility of bone marrow stem cells. Whether more specific subsets of bone marrow stem cells, such as MSCs (Hare et al. 2009), will be less, equally or more effective remains to be demonstrated. In addition, mechanisms of bone marrow cell-based cardiac repair remain to be defined.

Mouse studies have demonstrated that bone marrow-derived cells have the capacity to stimulate endogenous repair via CPC activation (Loffredo et al. 2011). Using a genetic approach, this study showed activation of pro-regenerative processes in resident CPCs after bone marrow-derived c-kit cell application. This effect was not observed when bone marrow-derived MSCs (c-kit negative) were employed. Notably, transdifferentiation of exogenously delivered cell was not detected, indicating that the c-kit cells elicited a regenerative response via the release of so far undefined factors. Other studies identified putative bone marrow-derived factors regulating myocardial regeneration from endogenous progenitor cells such as

fibroblast growth factors, stromal cell-derived factor 1 (Urbich et al. 2005), hepatocyte growth factor and insulin-like growth factor-1 (Gnecchi et al. 2006). Collectively, these data provide compelling evidence for the paracrine hypothesis of cell-based heart repair (Steinhauser and Lee 2011) and suggest that cell-free pharmacological interventions simulating cell-based paracrine effects may render pharmacoregeneration of the heart an attractive therapeutic option.

25.2.2 Endogenous Regenerative Capacity of the Heart

Two different mechanisms of endogenous cardiomyocyte generation have been suggested. These include (1) proliferation of pre-existing cardiomyocytes, which may necessitate initial cardiomyocyte dedifferentiation, and (2) provision of new cardiomyocytes from endogenous progenitor cell pools (Steinhauser and Lee 2011).

Diverse experimental evidence concerning cell cycle activity of cardiomyocytes in the adult heart has been reported (Soonpaa et al. 1996; Kajstura et al. 1998, 2010; Bergmann et al. 2009), ranging from annual proliferation rates of 0–20%. The quantitative disagreement could at least in part be attributed to the difficulty to unambiguously distinguish cardiomyocyte from non-myocyte nuclei and karyokinesis from cytokinesis. Despite this discrepancy, there is clear evidence that cardiomyocytes can in principle be coaxed into cell cycle progression for example by overexpression of large T antigen (Field 1988) and cyclin D2 (Pasumarthi et al. 2005). The latter study is particularly interesting because it demonstrated that cardiomyocyte restricted cyclin D2 overexpression would only enhance cardiomyocyte proliferation after infarction in the infarct border zone. This disease specific and spatially restricted activity of the cell cycle regulator cyclin D2 suggests that myocardial infarction and/or post-infarct remodeling create a unique myocyte growth supporting environment.

Recent experimental studies suggest that cardiomyocyte proliferation is preceded by cardiomyocyte dedifferentiation. For example, in zebrafish resection of cardiac tissue caused sarcomere disassembly and re-expression of so called fetal genes, followed by DNA synthesis leading to cytokinesis and re-differentiation toward mature cardiomyocyte (Jopling et al. 2010; Kikuchi et al. 2010). Interestingly, similar observations were made in the early neonatal mouse heart (Porrello et al. 2011). In agreement with the data mentioned above, these findings suggest that cardiomyocytes can undergo division under defined circumstances. Oncostatin M has recently been suggested as a specific paracrine mediator of cardiomyocyte dedifferentiation post myocardial injury (Kubin et al. 2011). Collectively, these data suggest that cardiomyocyte dedifferentiation and proliferation are closely interrelated. A better mechanistic understanding of these processes may offer novel approaches for pharmacoregeneration of the heart.

The identification of cells with progenitor cell properties in the heart raises the possibility that these cells can in principle be activated under specific post-injury cardiac milieu conditions to regenerate lost myocardium. However, quantity and

functionality of CPCs appears to decrease with age (Bergmann et al. 2009; Zaruba et al. 2010). The age associated deficit in cardiac regeneration capacity may contribute to heart failure progression in aging individuals. Amplifying and rejuvenating CPC niches in the heart are presently attempted by autologous CPC implantation (Bolli et al. 2011; Makkar et al. 2012). Direct pharmacological reactivation and amplification of endogenous CPC function appear equally attractive, and thus pharmacological means to achieve this are emerging. The following sections summarize the so far identified cardiac progenitor cell types in the heart. It is important to note that it cannot be excluded that these cell types are subsets of the same cell pool or derivatives from a common precursor.

25.2.3 Resident Cardiac Progenitor Cells

A number of cells with progenitor cell characteristics have been identified in the adult mature heart (Dimmeler et al. 2005; Laflamme and Murry 2011). Unlike in pre-natal progenitor cells, there is no consensus on the molecular identity of these populations (Steinhauser and Lee 2011) and moreover, there are no specific markers for selecting the different cell pools. The most common isolation protocols involve depletion of "contaminating" myocytes, smooth muscle, and endothelial cells followed by subsequent surface marker selection. The selection is typically based on expression of developmentally important genes such as islet1 (Laugwitz et al. 2005), specific cell surface receptors such as c-kit and Sca1 (Beltrami et al. 2003; Oh et al. 2003), the ability to form cell clusters termed "cardiospheres" (analogue to neurospheres) (Messina et al. 2004), or the property to efflux Hoechst dye via the transport protein Abcg2 (so called side population (SP) cells; (Martin et al. 2004)). Recently, additional cell populations were identified including the cardiac neural crest-derived cells (Tomita et al. 2005), epicardial cells expressing the Wilm's tumor 1 (Wt1) protein, and a population of adult cardiac-resident colony-forming unit – fibroblasts (cCFU-Fs) of epicardial origin (Chong et al. 2011). Although it has been proposed that these cells constitute distinct entities, there is the possibility that these populations overlap and that marker expression may differ in the same cell lineage during different stages of maturation (Laflamme and Murry 2011). Moreover, the origin of these cells remains still unclear and several hypotheses have been proposed including derivation from bone marrow or early hematopoietic cells, remnant cells from embryonic heart development, or derivation by endothelial-to-mesenchymal transformation (Dimmeler et al. 2005; Zeisberg et al. 2007). The following paragraphs summarize the to our knowledge most prominent CPC phenotypes:

25.2.3.1 Stem Cell Antigen 1 (Sca1 or Ly-6A/E) Positive Cells

Sca1 is a glycosylphosphatidylinositol-anchored membrane protein, which is expressed by immature hematopoietic progenitor cells and also found in a small

number of cardiac cells (Oh et al. 2003). Sca1 cells are negative for c-kit and do not express hematopoietic or stem or endothelial progenitor cell markers, but they exhibit high telomerase activity and express cardiogenic factors suggesting a cardiac pre-determination. Accordingly, *in vitro* differentiation of Sca1 cells towards cardiomyocyte-like cells has been documented upon treatment with 5′azacytidine (5aza), an inhibitor of DNA methyltransferase causing chromatin relaxation (Oh et al. 2003). Cardiac differentiation of Sca1 cells depends at least in part on bone morphogenetic proteins receptor (*Bmpr)1a*, which is a known regulator of heart development (Oh et al. 2003). *In vivo* these cells home to injured myocardium, integrate and differentiate when administered intravenously following ischemia-reperfusion injury (Oh et al. 2003). In this model, differentiation was found to be a result of two events: direct donor differentiation and cell fusion with the host tissue. Notably, the Sca1 epitope does not exist in the human. Despite this, Sca1-antibody selection has been successfully employed to isolate a cardiogenic cell type from fetal and adult human heart with similar cardiogenic properties as mouse Sca1 cells (Smits et al. 2005; van Vliet et al. 2008). Moreover, these cells also differentiate into functional cardiomyocytes *in vitro* in response to 5aza-mediated DNA demethylation and TGFbeta1 supplementation (Smits et al. 2005; Goumans et al. 2007). Further characterization of the biological properties of these Sca1 and Sca1-like cell populations is warranted to establish their therapeutic capacity in mice and men.

25.2.3.2 Tyrosine-Protein Kinase Kit (c-kit or CD117) Positive Cells

c-Kit is a tyrosine-kinase receptor found in circulating hematopoietic progenitors as well as in the bone marrow, telocytes, thymic epithelium, mast cells, and embryonic stem cells (Yasuda et al. 1993; Reber et al. 2006). c-Kit is also expressed in the CPC pool from the adult heart. Similar to Sca1-CPCs, c-kit-CPCs do not co-express hematopoietic markers; they are moreover clonogenic, self-renewing, and multipotent (Beltrami et al. 2003; Tallini et al. 2009). The cardiomyogenic capacity of c-kit cells is however highly disputed. Some groups reported the ability of c-kit cells to regenerate adult myocardium and vessels along with functional improvement after cardiac infarction of the adult heart (Beltrami et al. 2003; Bearzi et al. 2007); others report poor cardiomyogenic differentiation only in neonatal c-kit cells and no transdifferentiation in adult cardiac c-kit cells (Zaruba et al. 2010). Data from a BAC transgenic mouse model, which expressed GFP under the transcriptional control of the endogenous genomic c-kit locus, confirmed that cardiac c-kit cells can be identified in a mixed developmental state in the developing as well as the neonatal heart and observed a transcriptional c-kit reactivation in adult cardiomyocytes following injury. This study also showed the potential role of c-kit in vascular repair (Tallini et al. 2009) and is in agreement with studies showing the involvement of *in vitro* activated cardiac c-kit cell primarily in vasculogenesis post-infarction, but not in the absence of tissue damage (Tillmanns et al. 2008). Collectively, the comprehensive data in support of the utility of c-kit CPCs in heart regeneration has prompted the initiation of the SCIPIO trial to investigate the safety of intracoronary

autologous cardiac stem cell therapy as an adjunct treatment for patients with ischemic cardiomyopathy (Bolli et al. 2011). Early data from this phase I trial confirmed feasibility and safety of the stem cell approach. In addition, hints for improvement of left ventricular systolic function and reduction in infarct size could be attained. Larger clinical trials are necessary to ultimately define the therapeutic value of autologous c-kit CPCs.

25.2.3.3 Islet1-Positive Cells

Islet-1 is a transcription factor of the LIM-homeodomain family, which was first identified in cells of the pancreas as an enhancer of the insulin gene (Karlsson et al. 1990). Islet-1 raised interest in the field of cardiovascular research after the discovery that Islet-1 marks a cell population that makes a substantial contribution to the embryonic heart, i.e. mainly to the right ventricle, atria, the outflow tract, most of the conduction system, and also specific regions of the left ventricle (Cai et al. 2003; Laugwitz et al. 2005). Detailed *in vitro* studies demonstrated that Islet-1 positive cells from the heart can give rise to cardiomyocyte, endothelial cells, and smooth muscle cells (Laugwitz et al. 2005; Moretti et al. 2006). These observations positioned Islet-1 as a putative marker for resident CPCs. However, only few Islet-1 cells persist in the adult heart (Genead et al. 2010; Khattar et al. 2011). Using a genetic approach clusters composed of Islet-1 positive cells were confined to the sinoatrial node, cardiac ganglia, proximal aspects of the aorta, the pulmonary artery, and the outflow tract area (Weinberger et al. 2012). Given these data, and despite unequivocal evidence for the role of Islet-1 in prenatal mouse heart development (Cai et al. 2003), there is little evidence that Islet-1 is a unique marker for CPCs in the adult human heart.

25.2.3.4 Cardiosphere-Derived Cells (CDCs)

From myocardial biopsies, cells with the capacity to form spherical aggregates, also known as cardiospheres, can be derived (Messina et al. 2004). Cardiospheres can be generated from embryo, fetal, and postnatal mouse as well as explanted human atrial or ventricular biopsy specimens (Messina et al. 2004; Johnston et al. 2009). From cardiospheres self-renewing and clonogenic cells can be derived. Cardiosphere derived cells (CDCs) are Flk-1 positive, which is expressed in early cardiac mesodermal cells and hemangioblasts, along with the endothelial markers CD31 and CD34 as well as c-kit and Sca1. The origin of CDCs, i.e. either from dedifferentiated proliferative cardiomyocytes or CPCs, is unclear (Rasmussen et al. 2011). CDCs injected into the adult injured pig heart induced tissue repair and regeneration attenuating adverse remodeling post-infarction. This effect was attributed to the ability of CDCs to engraft and form mature cardiac cells (Barile et al. 2007; Johnston et al. 2009). In addition, CDCs appear to exhibit a unique paracrine activity, with the capacity to produce for example fibroblast and hepatocyte growth factors (Barile

et al. 2007). This may have contributed to their greater benefit on cardiac function when compared to bone-marrow derived cell grafts (Li et al. 2012). These results led to the initiation of a clinical study (CADUCEUS trial) to test the feasibility and safety of intracoronary injection of these cells after myocardial infarction (Makkar et al. 2012). Preliminary data from this study provided hints for therapeutic efficacy with smaller infarct size after CDC treatment. Also here larger clinical studies are necessary to unequivocally define the therapeutic potential of CDCs and underlying mechanisms for myocardial regeneration.

25.2.3.5 Side Population (SP) Cells

A subgroup of cells within the Sca1 cell fraction, also known as side population (SP), can be defined by their propensity to actively export Hoechst 3342 via verapamil sensitive Abcg2 efflux pumps (Zhou et al. 2001; Bunting 2002). SP cells can be isolated from multiple adult tissues including skeletal muscle, bone marrow, liver, lung, kidney, and brain. SP cells could also be identified in the early developing and adult heart tissue where they appear to contribute to organogenesis and tissue maintenance, respectively (Martin et al. 2004). A Sca1 positive SP subpopulation, being negative for the endothelial marker CD31, was identified to have a unique potential to differentiate into functional cardiomyocytes (Pfister et al. 2005, 2010). This appeared to depend on direct coupling with adult cardiomyocytes, although the exact mechanism requires further elucidation. Characterization of the SP cell population demonstrated their capacity to form aggregates resembling cardiospheres and differentiate into neurons, glia as well as smooth muscle cells (Tomita et al. 2005). It appeared that the cardiosphere forming cells exhibit characteristics of embryonic neural crest-derived cells.

25.2.3.6 Epicardial-Derived Cells (EPDCs)

The epicardium has recently moved into the focus of myocardial regeneration as a putative source for multiple cell types in the developing and adult heart (Smart et al. 2007, 2011). Its embryonic origin is the proepicardial organ (PEO) or proepicardium primordium. The PEO is a transient mesothelial cell cluster located near the venous pole of the embryonic heart that migrates and expands onto the myocardium to cover almost the whole surface of the ventricle (Ratajska et al. 2008). It is marked by the expression of the Wilm's tumor (WT-1) suppressor gene (Perez-Pomares et al. 2002). The epicardium provides epicardial cells that proliferate, undergo epithelial-to-mesenchymal transformation, and differentiate into endothelial and smooth muscle cells that constitute the coronary vasculature, interstitial fibroblast as well as the Purkinje fiber network (Winter and Gittenberger-de Groot 2007; Ratajska et al. 2008). Using reporter mice, WT-1 cells were found to contribute to cardiomyocyte formation during normal heart development (Zhou et al. 2008). Collectively, these studies showed the high plasticity of EPDCs, making them an

attractive population for cell-based cardiac repair. Moreover, the epicardium is also a source of diffusible factors required for development (Limana et al. 2011). Interestingly, epicardium-based myocardial repair activity in the mouse appears to be present only after preconditioning with thymosin beta 4 (Smart et al. 2011) and absent under "normal" pathological conditions (Zhou et al. 2012). Whether the human epicardium contains a pathophysiologically relevant reservoir of repair cells or exhibits paracrine regeneration-inducing activity remains to be demonstrated.

Taken together, experimental and clinical evidence suggests that the heart has an intrinsic, but dormant regenerative capacity. Especially in the aging heart endogenous repair appears to be lost. Reactivation of embryonic mechanisms controlling cardiomyocyte proliferation or CPC niche activity by cell-based or even better pharmacologically defined means would be highly attractive given the scarcity of alternatives in end-stage heart failure treatment.

25.3 Pharmacological Activation of Endogenous Cardiac Regeneration

Numerous studies have pointed to a prominent role of paracrine factors in cell-based cardiac regeneration. In fact, cardiomyocyte proliferation and endogenous CPC activity may be fully controlled by a regeneration supportive (typically present during prenatal development) or restrictive (typically present during postnatal development) paracrine milieu. Overcoming the restrictions in the adult heart by making use of pharmacological principles would be highly desirable and clearly preferred over the use of difficult to standardize cell therapy. Figure 25.1 summarizes potential pharmacological interventions discussed below in more detail. We would like to emphasize that the mechanisms of the regenerative action of the discussed growth factors/peptides and small molecules are only incompletely understood. The field is advancing rapidly to address the prevailing uncertainties.

25.3.1 Growth Factors/Peptides

25.3.1.1 Granulocyte and Macrophage Colony Stimulating Factor (GM-CSF)

GM-CSF is a cytokine responsible for the mobilization of hematopoietic stem cells. GM-CSF is produced by a variety of tissues and its well known function is to drive the proliferation of BMCs via activation of its canonical receptor (GM-CSFR). This is followed by activation of JAK/STAT, Ras/MAPK, and PI3K/AKT signaling cascades. GM-CSFR has also been detected in adult cardiomyocytes and its activation under stress seems to promote cell survival (Harada et al. 2005). Early studies by

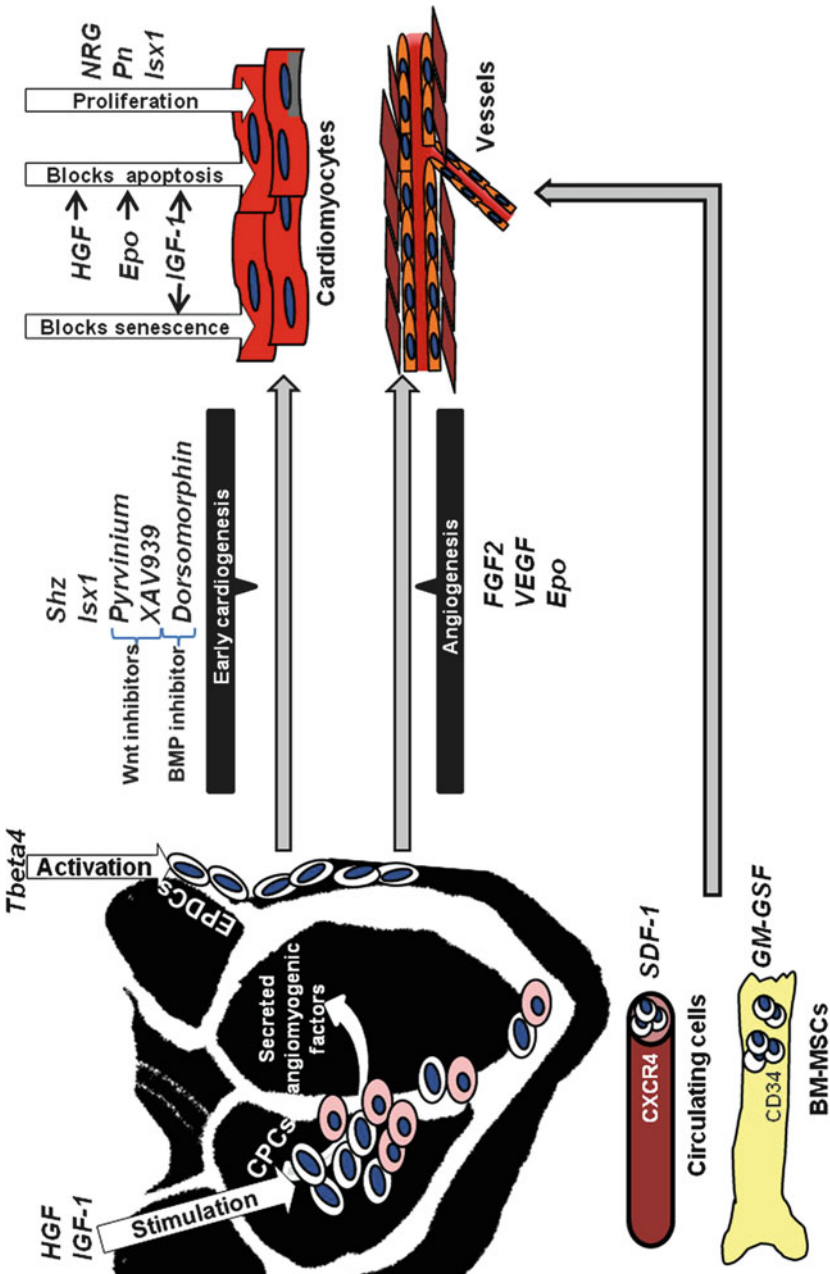


Fig. 25.1 Schematic overview of pharmacological interventions to regenerate the heart

Orlic at al. explored the systemic application of GM-CSF after myocardial infarction in a mouse model (Orlic et al. 2001a, b). They found recruitment of c-kit-BMCs and attributed the observed reduction in infarct size in the GM-CSF treated group to stem cell transdifferentiation into myocytes and endothelial cells. This mechanism of action has been clearly refuted by a number of elegant studies (Balsam et al. 2004; Murry et al. 2004), but the therapeutic potential of GM-CSF (not its originally postulated mechanism) has been further substantiated (Sugano et al. 2005; Hasegawa et al. 2006). In line with these data, the first clinical trial using GM-CSF in combination with intracoronary infusion of peripheral blood stem cells (PBMCs; MAGIC trial) showed an improvement in cardiac function and promotion of angiogenesis post-infarction (Kang et al. 2004). However, aggravated coronary restenosis observed in the patients receiving GM-CSF raised serious safety concerns and the patient enrollment was consequently terminated. In contrast to MAGIC, no coronary restenosis was observed in a follow-up trial (FIRSTLINE-AMI), where GM-CSF alone was administered to patients with myocardial infarction (Ince et al. 2005). Analyses revealed mobilization of CD34-BMCs along with significant improvement in cardiac contractility. The results from FIRSTLINE-AMI encouraged subsequent trials with a higher number of patients as well as placebo controls (STEMMI, REVIVAL-2, G-CSF-STEMI). These trials, however, did not show any beneficial effect of GM-CSF post infarction (Engelmann et al. 2006; Ripa et al. 2006; Zohlnhofer et al. 2006). Nevertheless it should be noted that in these studies GM-CSF did not elicit adverse effects such as coronary restenosis, aggravation of inflammation or ischemia. Moreover, a recent meta-analysis of these trials revealed a significant amelioration in cardiac function (increase in left ventricular ejection fraction [LVEF] by 4.7%) when GM-CSF was administered within 37 h post ischemia (Abdel-Latif et al. 2008). In conclusion, regardless of the diverse experimental and clinical results, GM-CSF seems to exert a modest therapeutic effect if administered shortly after acute ischemia. The GM-CSF effect in acute phases of myocardial injury argues for a cell protective effect, which could be mediated via anti-apoptotic AKT-signaling. However, activation of myocardial regeneration by induction of cardiomyocyte proliferation and/or CPC activation may also have contributed to the observed effects.

25.3.1.2 Stromal Cell-Derived Factor 1 (SDF-1)

SDF-1 is a small chemokine of 8 kDa that by binding to C-X-C chemokine receptor type 4 (CXCR4) has originally been identified to chemoattract CD34-BMCs (Aiuti et al. 1997). Embryos lacking either SDF-1 or its receptor die due to impaired myelopoiesis (Zou et al. 1998). In addition, SDF-1 null embryos show cardiac ventricular septum defects suggesting that SDF-1/CXCR4 may play a distinct role during cardiogenesis (Nagasawa et al. 1996). In line with this, gene knock down of CXCR4 in pluripotent stem cells abrogated their spontaneous differentiation into functional cardiomyocytes (Chiriac et al. 2010). Together, these data suggest that the SDF-1/CXCR4 axis does not only induce progenitor cell migration in the heart, but also promotes lineage-specific differentiation. Under the hypothesis that SDF-1

could promote cardiogenesis after myocardial infarction in a similar way as in development, Askari et al. transplanted stably over-expressing SDF-1 fibroblasts in the ischemic rat heart (Askari et al. 2003). Here, high levels of SDF-1 resulted in a higher vessel density and improved cardiac function. Moreover, SDF-1 was shown to be expressed by endothelial cells in the ischemic heart under the control of hypoxia-inducible factor-1 (Ceradini et al. 2004). SDF-1 expression increased migration and homing of circulating CXCR4-positive progenitor cells to ischemic tissue whereas in the absence of SDF-1 or CXCR4 no progenitor cell recruitment was observed. Although, SDF-1 is activated under hypoxia, its clinical potential against cardiac remodeling is limited by its rapid degradation by proteases present in the ischemic heart such as matrix metalloproteinase MMP-2 (McQuibban et al. 2001) and dipeptidylpeptidase IV (DPP IV/CD26) (De La Luz Sierra et al. 2004). In an initial approach to overcome this problem, a cleavage protected variant of SDF-1, called SSDF-1(S4V), was designed. Delivery of SSDF-1(S4V) with self-assembling peptide nanofibers achieved high and locally sustained concentrations; attributed to this was increased cardiac function and vessel density in a rat model of cardiac ischemia (Segers et al. 2007). An alternative approach to increase SDF-1 is by DPP IV inhibition. This resulted in CD34-BMCs recruitment to the infarct border zone and enhanced micro-vascularization; both may have contributed to the observed reduction in infarct scar size (Zaruba et al. 2009). A combination with GM-CSF-based stem cell mobilization further enhanced survival and cardiac function (Zaruba et al. 2009). Non-viral gene transfer of naked plasmid DNA encoding for human SDF-1 promoted angiogenesis and improved cardiac function in rats with ischemic heart failure (Sundararaman et al. 2011). These studies demonstrated collectively that SDF-1 attenuates the progression of chronic ischemic heart failure primarily by increasing vasculogenesis, reducing scar formation and fibrosis. A clinical trial which investigates the effects of SDF-1 in myocardial ischemia has been initiated (ClinicalTrials.gov Identifier: NCT01082094). This trial evaluates the safety of a single escalating dose of SDF-1 plasmid administered by endomyocardial injection. Taken together, SDF-1 appears as a promising therapeutic agent to minimize post infarct injury and may thereby prevent progression into heart failure. Similar as for GM-CSF it remains to be established whether protection from ischemia inflicted damage or true regeneration are the underlying therapeutic mechanisms.

25.3.1.3 Hepatocyte Growth Factor (HGF)

HGF was originally identified as a cytokine acting in hepatocytes (Nakamura et al. 1989) by binding with high affinity to its receptor c-met. c-Met is expressed in a variety of cells, which upon HGF challenge can proliferate, migrate, and form tubules (Derman et al. 1995). Interestingly, c-met is expressed on different populations of progenitor cells including c-kit-cells (Beltrami et al. 2003; Miyazaki et al. 2004), Sca1-cells (Iwasaki et al. 2005) and mesenchymal stem cells (MSCs) (Forte et al. 2006). Following ischemia/reperfusion, c-met expression is induced in the ischemic rat heart (Nakamura et al. 2000). Recombinant HGF has been shown to reduce scar

size by protecting myocytes from apoptosis whereas HGF neutralization did the opposite. Besides its anti-apoptotic role, HGF in combination with insulin like growth factor-1 (IGF-1) has been shown to recruit c-kit-positive CPCs in the ischemic heart (Urbanek et al. 2005). The in this study observed amelioration in cardiac function was attributed to the differentiation of c-kit-positive cells into cardiomyocytes as well as endothelial cells in the infarct border zone and epicardium (Wang et al. 2004). The anti-apoptotic properties of HGF seem to be mediated via the activation of PI3-kinase/Akt pathway. A similar property is attributed to IGF-1 and PDGF-BB (Vantler et al. 2010). Although HGF administration in preclinical trials provided promising results, we are not aware of a clinical study that assesses the effect of HGF against ischemia.

25.3.1.4 Insulin-Like Growth Factor-1 (IGF-1)

IGF-1 is typically released from a variety of cells after growth hormone stimulation. A surplus in IGF-1 is associated with acromegaly, which may cause cardiac hypertrophy (Bogazzi et al. 2008). Approximately 98% of IGF-1 is bound to one of the seven binding proteins (IGF-BP). IGF-1 binds to its tyrosine kinase receptor, the insulin like growth factor 1 receptor (IGF1R), and thereby triggers PI3K/AKT activation with its well known supportive effects on cell growth, i.e. hyperplasia and hypertrophy, and cell survival. The hypertrophy inducing effects of IGF-1 formed the rationale for clinical trials testing IGF-1 administration in patients with dilated cardiomyopathy (Fazio et al. 1996). However, these early trials had a neutral outcome, and thus further testing was abandoned. Additional academic interest was raised by the *in vivo* evidence of the IGF1/IGF1R system being activated in cardiomyocytes shortly after myocardial infarction (Anversa et al. 1995). Moreover, constitutive overexpression of IGF-1 reduced apoptosis at the infarct border zone while limiting adverse cardiac remodeling (Li et al. 1997). Aside from its anti-apoptotic effects, IGF-1 was also implicated in angiomyogenic regeneration (Hynes et al. 2011). More recently, IGF-1 was identified as a key regulator of cellular senescence (Torella et al. 2004). Accordingly, in IGF-1 overexpressing mice transcription of senescence genes p27Kip1, p53, p16INK4a, and p19ARF was repressed. Moreover, IGF-1 overexpressing mice exhibited high nuclear phospho-Akt and telomerase activity particularly in cardiomyocytes and c-kit-positive cells in the heart, favoring cardiac regeneration (Torella et al. 2004). Interestingly, also in humans an age-related inverse relationship between senescence and IGF could be identified (Reeves et al. 2000). It is consequently intriguing to speculate that there is a causal link between loss of IGF-1 and CPC quantity as well as activity during aging and that reintroduction of "juvenile" IGF-1 levels would have the capacity to antagonize age-associated myocardial deterioration. The link between IGF-1 and CPCs was further substantiated by studies showing that c-kit-positive CPCs express IGF-1R and also synthesize IGF-1, suggesting an autocrine mechanism (D'Amario et al. 2011). Full activity of this putative positive feedback mechanism may however depend on the availability of HGF (Rota et al. 2008; Padin-Iruegas et al. 2009). In addition to its direct effects on CPC fate, IGF-1 may also protect/regenerate the

heart by inducing the secretion of other protective or regenerative factors such as SDF-1 (Haider et al. 2008) or modulation of micro RNA processing (e.g. miR-34a), which can confer anti-apoptotic effects (Iekushi et al. 2012). Very recently, a clinical trial (RESUS-AMI; ClinicalTrials.gov Identifier: NCT01438086) has been initiated to test safety and preliminary efficacy of IGFR activation in the heart by applying a synthetic IGF-1 analog (i.e. mescasermin) in acute ischemia.

25.3.1.5 Fibroblast Growth Factor (FGF)/Vascular Endothelial Growth Factor (VEGF)

Angiogenesis is closely linked to myocardial repair or regeneration. Growth factors with well documented angiogenic activity include fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). Their role in direct cardiomyocyte regeneration is less well defined. In embryonic development, FGFs have a pivotal role in the control of early cardiogenesis (Watanabe et al. 2006; Grego-Bessa et al. 2007). Similarly, VEGF is essential for embryonic vessel formation, stabilization, and remodeling via regulation of NOTCH signaling (Lin et al. 2007). Also in the adult heart, a combination of FGF-2 and VEGF induced endothelial cell proliferation and angiogenesis, resulting in attenuation of post infarction injury (Yanagisawa-Miwa et al. 1992; Pearlman et al. 1995; Watanabe et al. 1998). Despite the promising results of the preclinical animal studies, safety concerns about the mitogenicity of these factors and the broad expression of their receptors restricted their systemic administration in humans. To circumvent systemic delivery of angiogenic growth factors, which would facilitate the risk of tumor formation, a mutated non-angiogenic form of FGF-2 (S117A-FGF-2 (Jiang et al. 2004)) and targeted VEGF delivery via P-selectin coated liopsomes (Scott et al. 2009) were tested and demonstrated protection of infarcted myocardium from myocyte loss and adverse remodeling. Despite these encouraging data, it has become apparent that for functional vascularization not only endothelial cells, but also smooth muscle cells or pericytes are required. Thus, additional growth factors may have to be administered to ensure proper vessel formation. Despite these caveats, clinical tests with combined VEGF/FGF administration via intramyocardial injection of plasmid-DNA (pVIF) in patients with refractory cardiac ischemia (VIF-CAD) have been performed (Kukula et al. 2011). Although the primary study endpoint, i.e. enhancement of myocardial perfusion, was not reached, patients did show better exercise tolerance. Using an alternative approach, the ALCADIA study is recruiting to evaluate the safety and efficacy of autologous human CPCs transplantation in combination with controlled release of FGF-2 in a gelatin hydrogel sheet in patients with chronic ischemic cardiomyopathy (ClinicalTrials.gov Identifier: NCT00981006).

25.3.1.6 Erythropoietin (Epo)

Epo is a 34 kDa growth hormone, widely known for its role in erythropoiesis. It was originally identified to enhance survival and induce proliferation of the late erythroid

progenitors, colony forming unit-erythroid (CFU-E) (Gregory and Eaves 1978; Komatsu et al. 1991). However, recent data on the broad expression of erythropoietin receptor (EpoR) in adult organs (Suzuki et al. 2002) suggested a comprehensive role for Epo beyond erythropoiesis. Specifically in the heart, EpoR is detected around embryonic stage E10 and although significantly lower it persists through adulthood. Erythroid rescued EpoR knock-out mice appear to have less immature proliferating myocytes during embryogenesis, but reach adulthood without apparent morphological defects (Suzuki et al. 2002). However, upon ischemia reperfusion, these animals show a greater infarct size suggesting that a deficient Epo/EpoR system in the non-hematopoietic lineage may deteriorate left ventricular remodeling (Tada et al. 2006). Epo expression is controlled by HIF-1alpha, suggesting a central role in ischemic tissue (Makita et al. 2005). In agreement with this, several preclinical studies showed that Epo administration post infarction improves cardiac function mainly via neoangiogenesis and anti-apoptotic mechanisms (Calvillo et al. 2003; Moon et al. 2003; Hirata et al. 2005; Xu et al. 2005; Ye et al. 2005). Owing to its approved availability for anemia therapy, Epo was quickly tested clinically for its utility in post myocardial infarction repair. In the first pilot study, a high i.v. single dose Epo regimen was able to induce endothelial cell proliferation, but had no effect on cardiac function (Lipsic et al. 2006). Subsequent studies with larger numbers of patients also failed to show a significant amelioration in cardiac function (Ott et al. 2010). Nevertheless, a smaller pilot study where small amounts of Epo were administered subcutaneously once a week in patients for a prolonged period of time demonstrated that Epo had a beneficial effect on cardiac remodeling and function (Bergmann et al. 2011). These data suggest that the discrepancy between the promising pre-clinical results of Epo and the human trials may be due to differences in dosing and mode of delivery (intravenously vs. subcutaneously). In this line of thought, a novel clinical trial (EPAMINONDAS; EudraCTno. 200500485386) is about to establish the optimum concentration of intravenous Epo injection in a large cohort of patients with myocardial infarction. In another study, biological half-life of Epo was found to be markedly different if the same dose of Epo was administered intravenously and subcutaneously, i.e. ~5 vs. ~25 h, respectively (Salmonson et al. 1990). Collectively, these data suggest that a slow release application of Epo could be advantageous. The high receptor affinity (Kd 160 pM) would moreover only necessitate low doses to activate the EpoR effectively (Syed et al. 1998). Thus, it appears timely to test the potential role of subcutaneous low dose of Epo in ischemia and define in more detail its role not only in angiogenesis, but also in CPC activation or cardiomyocyte proliferation.

25.3.1.7 Thymosin Beta 4 (Tbeta4)

Tbeta4 is a highly conserved 5 kDa peptide initially isolated from bovine thymus (Low et al. 1981) that forms a complex with muscle G-actin and inhibits its polymerization (Safer et al. 1991). Tbeta4 ability to induce both ectoderm and mesoderm commitment of P19 embryonic cells and its localization in blood vessels and heart of developing embryos, suggested a role in stem cell biology and potentially angiogenesis (Gomez-Marquez et al. 1996). Indeed, TBeta4, which is also a

direct downstream target of Hand 1 (Smart et al. 2010), was shown to promote myocardial and endothelial cell migration in the embryonic and early postnatal heart (Bock-Marquette et al. 2004). In the first preclinical trial, Tbeta4 treatment of infarcted mice led to an improvement in cardiac function as well as cardiomyocyte survival; this action was mediated by integrin-linked kinase (ILK) and subsequent Akt activation (Bock-Marquette et al. 2004). Further studies revealed that Tbeta4 is a prerequisite for coronary vessel development in mice and that upon injury it may partially restore the lost vasculature not only by activating quiescent epicardial progenitor cells, but also by inducing their differentiation into fibroblasts, smooth muscle, and endothelial cells (Smart et al. 2007). In a large study, Tbeta4 was identified as a paracrine factor that mediated cardioprotection when embryonic endothelial cells where injected into porcine infarcted myocardium (Hinkel et al. 2010). Finally, a recent study showed that upon myocardial injury WT1-positive EPDCs primed with Tbeta4 were able to transdifferentiate into and integrate with functional cardiomyocytes (Smart et al. 2011). However, being an apparently mitogenic peptide Tbeta4 administration in humans needs to be carefully performed to not cause tumor formation or unknown tumor aggravation (Jo et al. 2011). Therefore novel injectable hydrogels are being designed that are able not only to target Tbeta4 to the heart, but also ensure its sustained delivery (Chiu and Radisic 2011; Kraehenbuehl et al. 2011). Collectively, these promising results about Tbeta4-based cardioprotection led to the first placebo controlled clinical trial, testing intravenous administration of Tbeta4 in patients with acute myocardial infarction. Phase I testing has recently been completed with encouraging results and phase II clinical trials are under preparation (Crockford 2007).

25.3.1.8 Emerging Myocyte Mitogens – Periostin and Neuregulin

One of the first exogenous proteins used to mitotically activate myocytes was *periostin* (Pn). Pn is a 90 kDa protein that typically controls cellular and extracellular matrix organization during cardiac development (Butcher et al. 2007). Pn levels decrease substantially during aging and are up-regulated upon injury (Stanton et al. 2000). Pn knockout mice are more sensitive to cardiac rapture and fibrosis, but in the long-term exhibit hypertrophy and a better cardiac function (Oka et al. 2007). *In vitro*, Pn could be employed to stimulate mononucleated cardiomyocytes to proliferate, and in a rat model of myocardial ischemia delivery of a Pn foamgel resulted in neoangiogenesis, reduced infarct size and fibrosis, and improved cardiac function (Kuhn et al. 2007). However, later studies where Pn was transgenically overexpressed in mouse myocytes failed to validate these results (Fuller et al. 2008; Lorts et al. 2009). Whether the discrepancies between these studies can be explained by the different species used (mouse vs. rat), the mode of delivery of Pn, or the different experimental protocol remains to be clarified.

Neuregulin 1 (NRG) is another "myocyte mitogen" that has raised a lot of attention. The importance of NRG for proper cardiac development is demonstrated in mice with a NRG null mutation, which display heart malformations (Meyer and Birchmeier

1995). Alternative splicing of NRG1 results in a number of different isoforms, including heregulins (HRGs), glial growth factors (GGFs), and sensory and motor neuron-derived factors (SMDF). Most of these isoforms have been confirmed as inductors of proliferation and/or differentiation of cells expressing members of the epidermal growth factor receptor family ErbB1-4, including myocytes (Peles et al. 1992; Zhao et al. 1998; Fuller et al. 2008). NRG1- or ErbB-2 and -4 knockout mice die at mid-gestation due to myocardial hypotrophy (Gassmann et al. 1995; Lee et al. 1995; Meyer and Birchmeier 1995), depicting the significance of this gene in cardiac development. Postnatally, both ErbB2 and ErbB4 receptors were found in neonatal and adult ventricular myocytes (Fuller et al. 2008). They could be activated with recombinant human GGF 2 (Zhao et al. 1998). This activation promoted the survival of stressed myocytes and further led to their hypertrophy. Finally, the same authors revealed that the source of NRG1 in the rat ventricle are endothelial cells of the coronary microvasculature. Recently, NRG1 was shown to play a mitogenic role in adult mononucleated cardiomyocytes *in vitro* and *in vivo*, which apparently contributed to improved cardiac function post myocardial infarction (Bersell et al. 2009). Clinical trials to evaluate the safety and efficacy of NRG-1 have shown improved cardiac function and reversed remodeling in chronic heart failure (Xu et al. 2010). There are now several follow-up clinical trials recruiting patients to test safety and efficacy of NRG1 (or GGF2) in heart failure patients (NCT01439789, NCT01541202, NCT01251406, NCT01439893). Other trials are however on hold because of concerns related to unwanted cell growth (NCT01214096, NCT01131637).

25.3.2 Small Molecules

The introduction of small molecules with regeneration inducing activity would be highly advantageous over peptide based therapeutics, because of their defined chemistry and thus producibility under highly defined conditions. Moreover, small molecules are cheaper to produce in bulk quantities and require less regulatory considerations. Small molecules are usually developed based on identified therapeutic targets. This is followed by compound screens and chemical engineering based on for example the crystal structure of a putative therapeutic target. Especially, in compound screens it is essential to use adequate model systems, which simulate as closely as possible the target organ physiology and pathology. Here compromises are often made to achieve high throughput at low costs, e.g. by making use of transformed cell lines (P19CL6, HL-1) with little resemblance to *bona fide* cardiomyocytes. More recently human embryonic stem cell or induced pluripotent stem cell based models have been introduced to provide more realistic models (Davis et al. 2011). However, the validity of stem cell-based assays still needs to be confirmed. Despite these limitations, there is some evidence for the capacity of specific small molecules to facilitate cardiac regeneration. These compounds typically modulate signaling pathways involved in embryonic cardiogenesis, cell cycle control, myocardial remodeling, and survival. Some examples are described in the following paragraphs:

25.3.2.1 Sufonylhydrazone (Shz)

The first small molecule activating the early cardiac progenitor cardiac gene Nkx2.5 was found by Sadek et al. (2008). They designed a small-molecule library screen for chemical activators of Nkx2.5 driving the firefly luciferase in P19CL6 embryonic carcinoma cells and identified the molecule sufonylhydrazone (Shz). Shz was able to specifically activate expression of the pan-mesodermal marker brachyury-T along with a cardiac muscle- and smooth muscle- transcriptional activator, myocardin, in pluripotent stem cells. They tested the effect of Shz in PBMCs and found that attachment and survival of these cells was enhanced *in vitro*. Moreover, human PBMCs isolated from healthy patients and treated for 3 days with Shz were injected in the border zone of a cryo-injured rat heart. This resulted in normalization of contractile function 21 days after "Shz-enhanced PBMC" administration. The mechanism through which Shz regulated activation of a cardiac regeneration remains so far undiscovered. Contribution of Wnt, BMP, and FGF signaling was excluded, indicating that a non-redundant, but complementary regenerative pathway may have been activated by Shz.

25.3.2.2 *3,5*-Disubstituted Isoxazoles (Isx)

Using the same high-throughput screening system as described above, the cyclopropylamide analogue called Isx1, which belongs to the 3,5-disubstituted isoxazoles, was identified as "cardiogenic" (Russell et al. 2012). The effect of Isx1 was tested in a multipotent stromal cell population that dynamically responds to injury and participates in fibrosis repair of the adult heart. These cells are identified as a non-hematopoietic ($CD45^-$) and non-endothelial ($CD31^-$) Notch-activated epicardial-derived cells (NECs) (Russell et al. 2011). Daily intraperitoneal administration of Isx1 in mice resulted in robust activation of cardiac gene programs in multipotent NECs and cell cycle activity in myocardial cells. After myocardial ischemia, Isx1 administration initially improved ventricular function, but this effect was not anymore significant 21 days after injury. In this model Isx1 induced a distinct transcriptional program in NEC-fibroblasts, which included activation of genes implicated in angiogenesis, but excluded muscle gene activation. The authors of this study argued, that very strong fibrosis induced upon cardiac remodeling may have acted as a barrier for the Isx1-based cardiogenic effect. Further validation of the specific activity of Isx1 and optimization of its bioavailability are essential for its further exploitation in pharmacoregeneration.

25.3.2.3 Wnt/β-Catenin and BMP Signaling Inhibitors

Regulation of Wnts and BMPs in a highly temporally controlled manner is essential for proper specification and differentiation of cardiogenic cells during embryogenesis (Mercola et al. 2011). Wnts promote cardiogenesis during mesoderm induction, but act as inhibitors of committed cardiac progenitors (Schultheiss et al. 1997; Gessert and Kuhl 2010). In contrast, BMP2 and BMP4 inhibit cardiogenesis during mesoderm formation while reduced BMP signaling enhances dorsal cardiogenic

mesoderm (Yuasa et al. 2005; Hao et al. 2008). Given this background, Wnt and BMP modulation may turn out to be an attractive approach to promote tissue regeneration via cell cycle control or activation of resident CPCs.

Using high-throughput screening the small molecule, pyrvinium (FDA-approved as an anthelmintic drug), was classified as a Wnt signaling inhibitor. This inhibition is achieved by directly acting on a downstream casein kinase-1, which phophorylates β-catenin to target it for ubiquitination and subsequent degradation (Saraswati et al. 2010). The therapeutic effect of pyrvinium was tested in a mouse model of cardiac ischemia. A single intracardiac injection of pyrvinium reduced adverse cardiac remodeling along with stimulation of proliferation of pre-existing cardiomyocytes in peri-infarct and distal myocardium. Follow-up studies are needed to advance this innovative concept and address potential caveats associated with the unspecific modulation of Wnt signaling (i.e. mainly tumor formation).

Using a chemical screen, the small molecule *XAV939* was identified as a selective inhibitor of β-catenin-mediated transcription. XAV939 stimulates β-catenin degradation by stabilizing axin, which is part of the β-catenin destruction complex. This stabilization occurs via inhibition of the enzymes tankyrase 1 and 2 (Huang et al. 2009). The potential of this molecule to influence cardiogenesis was tested in mouse embryonic stem cells (mESCs). Application of XAV939 in mESCs resulted in robust stimulation of cardiomyogenesis in a time window coinciding with the initiation of mesoderm formation and specification (Wang et al. 2011). This stimulation occurred at the expense of other mesoderm derived lineages, including endothelial, smooth muscle, and hematopoietic lineages. The bioavailability of XAV939 or its structural analogous are expected to facilitate the exploration of its regenerative capacity also *in vivo* (Wang et al. 2011).

Dorsomorphin was identified as the first known small-molecule selectively inhibiting BMP type I receptors and therefore, blocking BMP-mediated SMAD1/5/8 phosphorylation (Yu et al. 2008). Similar to XAV939, pharmacological inhibition of BMP signaling during the initial stages of ESC differentiation appears to stimulate pre-cardiac mesodermal cells at the expense of endothelial, smooth muscle, and hematopoietic lineages (Hao et al. 2008). Dorsomorphin treatment in the initial 24 h of cell differentiation was sufficient to significantly boost cardiac induction, indicating its role on very primitive pluripotent cells.

Pyrvinium, XAV939, and dorsomorphin are just some examples for compounds that modulate the developmentally extremely important Wnt/β-catenin and BMP signaling pathways. The main challenge is to better understand the activity of these pathways in the adult heart and refine compounds as highly specific, efficient, and safe modulators of Wnt/β-catenin and BMP signaling in a relevant patient cohort.

25.4 Conclusion

Progenitor cells have been identified in various organs of the body, but vary in number and potential. The bone marrow is the classical source for circulating stem cells, which are typically attracted to tissue damage by cytokine signals released in

response to cell death, hypoxia, and/or inflammation. Resident CPCs are distinct from bone marrow cells and appear to occupy dormant niches with regenerative capacity in the heart. Number and reparative potential of resident CPCs are negatively correlated with age and disease. Unlocking their capacity to regenerate the heart by pharmacological means would be highly attractive in light of the limited therapeutic options in end-stage heart failure treatment. Increasing knowledge in cell fate control, cell proliferation, and differentiation in the mature heart will facilitate the identification of druggable targets in the failing heart. Early animal studies and clinical trials have tested emerging peptide and small molecule based approaches and provided compelling evidence for the novel concept of cardiac pharmacoregeneration. Further refinement of ideally chemical compounds and identification of their mechanism of action remain absolutely necessary to not only raise academic interest, but also excite the pharmaceutical industry to start to (re-)invest in the development of the next generation cardiovascular therapeutics.

References

Abdel-Latif A, Bolli R, Tleyjeh IM et al (2007) Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. Arch Intern Med 167(10):989–997

Abdel-Latif A, Bolli R, Zuba-Surma EK et al (2008) Granulocyte colony-stimulating factor therapy for cardiac repair after acute myocardial infarction: a systematic review and meta-analysis of randomized controlled trials. Am Heart J 156(2):216–226 e219

Aiuti A, Webb IJ, Bleul C et al (1997) The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med 185(1):111–120

Amado LC, Saliaris AP, Schuleri KH et al (2005) Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl Acad Sci USA 102(32):11474–11479

Anversa P, Reiss K, Kajstura J et al (1995) Myocardial infarction and the myocyte IGF1 autocrine system. Eur Heart J 16(Suppl N):37–45

Anversa P, Leri A, Rota M et al (2007) Concise review: stem cells, myocardial regeneration, and methodological artifacts. Stem Cells 25(3):589–601

Askari AT, Unzek S, Popovic ZB et al (2003) Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 362(9385):697–703

Balsam LB, Wagers AJ, Christensen JL (2004) Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 428(6983):668–673

Barile L, Chimenti I, Gaetani R et al (2007) Cardiac stem cells: isolation, expansion and experimental use for myocardial regeneration. Nat Clin Pract Cardiovasc Med 4(Suppl 1):S9–S14

Bearzi C, Rota M, Hosoda T et al (2007) Human cardiac stem cells. Proc Natl Acad Sci USA 104(35):14068–14073

We certainly failed to include all outstanding contributions to the emerging field of pharmacoregeneration and wish to apologize to the authors that we have missed undeliberately. Please, contact us to improve any follow-up versions of this book chapter. The authors are supported by the Deutsche Forschungsgemeinschaft (DFG) and Bundesministerium für Bildung und Forschung (BMBF) and are members of the Deutsches Zentrum für Herzkreislaufforschung (DZHK).

Beltrami AP, Barlucchi L, Torella D et al (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114(6):763–776

Bergmann O, Bhardwaj RD, Bernard S et al (2009) Evidence for cardiomyocyte renewal in humans. Science 324(5923):98–102

Bergmann MW, Haufe S, von Knobelsdorff-Brenkenhoff F et al (2011) A pilot study of chronic, low-dose epoetin-{beta} following percutaneous coronary intervention suggests safety, feasibility, and efficacy in patients with symptomatic ischaemic heart failure. Eur J Heart Fail 13(5):560–568

Bersell K, Arab S, Haring B et al (2009) Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. Cell 138(2):257–270

Bock-Marquette I, Saxena A, White MD et al (2004) Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. Nature 432(7016):466–472

Bogazzi F, Lombardi M, Strata E et al (2008) High prevalence of cardiac hypertophy without detectable signs of fibrosis in patients with untreated active acromegaly: an in vivo study using magnetic resonance imaging. Clin Endocrinol (Oxf) 68(3):361–368

Bolli R, Chugh AR, D'Amario D et al (2011) Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet 378(9806):1847–1857

Bunting KD (2002) ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells 20(1):11–20

Butcher JT, Norris RA, Hoffman S et al (2007) Periostin promotes atrioventricular mesenchyme matrix invasion and remodeling mediated by integrin signaling through Rho/PI 3-kinase. Dev Biol 302(1):256–266

Cai CL, Liang X, Shi Y et al (2003) Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell 5(6):877–889

Calvillo L, Latini R, Kajstura J et al (2003) Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. PNAS 100(8):4802–4806

Ceradini DJ, Kulkarni AR, Callaghan MJ et al (2004) Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 10(8):858–864

Chiriac A, Terzic A, Park S et al (2010) SDF-1-enhanced cardiogenesis requires CXCR4 induction in pluripotent stem cells. J Cardiovasc Transl Res 3(6):674–682

Chiu LL, Radisic M (2011) Controlled release of thymosin beta4 using collagen-chitosan composite hydrogels promotes epicardial cell migration and angiogenesis. J Control Release 155(3):376–385

Chong JJ, Chandrakanthan V, Xaymardan M et al (2011) Adult cardiac-resident MSC-like stem cells with a proepicardial origin. Cell Stem Cell 9(6):527–540

Crockford D (2007) Development of thymosin beta4 for treatment of patients with ischemic heart disease. Ann N Y Acad Sci 1112:385–395

D'Amario D, Cabral-Da-Silva MC, Zheng H et al (2011) Insulin-like growth factor-1 receptor identifies a pool of human cardiac stem cells with superior therapeutic potential for myocardial regeneration. Circ Res 108(12):1467–1481

Davis RP, van den Berg CW, Casini S et al (2011) Pluripotent stem cell models of cardiac disease and their implication for drug discovery and development. Trends Mol Med 17(9):475–484

Dawn B, Abdel-Latif A, Sanganalmath SK et al (2009) Cardiac repair with adult bone marrow-derived cells: the clinical evidence. Antioxid Redox Signal 11(8):1865–1882

De La Luz Sierra M, Yang F, Narazaki M et al (2004) Differential processing of stromal-derived factor-1alpha and stromal-derived factor-1beta explains functional diversity. Blood 103(7):2452–2459

Derman MP, Cunha MJ, Barros EJ et al (1995) HGF-mediated chemotaxis and tubulogenesis require activation of the phosphatidylinositol 3-kinase. Am J Physiol 268(6 Pt 2):F1211–F1217

Dimmeler S, Zeiher AM, Schneider MD (2005) Unchain my heart: the scientific foundations of cardiac repair. J Clin Invest 115(3):572–583

Engelmann MG, Theiss HD, Hennig-Theiss C et al (2006) Autologous bone marrow stem cell mobilization induced by granulocyte colony-stimulating factor after subacute ST-segment elevation myocardial infarction undergoing late revascularization: final results from the G-CSF-STEMI (Granulocyte Colony-Stimulating Factor ST-Segment Elevation Myocardial Infarction) trial. J Am Coll Cardiol 48(8):1712–1721

Fazio S, Sabatini D, Capaldo B et al (1996) A preliminary study of growth hormone in the treatment of dilated cardiomyopathy. N Engl J Med 334(13):809–814

Fernandez-Aviles F, San Roman JA, Garcia-Frade J et al (2004) Experimental and clinical regenerative capability of human bone marrow cells after myocardial infarction. Circ Res 95(7):742–748

Field LJ (1988) Atrial natriuretic factor-SV40 T antigen transgenes produce tumors and cardiac arrhythmias in mice. Science 239(4843):1029–1033

Forte G, Minieri M, Cossa P et al (2006) Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation. Stem Cells 24(1):23–33

Fuller SJ, Sivarajah K, Sugden PH (2008) ErbB receptors, their ligands, and the consequences of their activation and inhibition in the myocardium. J Mol Cell Cardiol 44(5):831–854

Gassmann M, Casagranda F, Orioli D et al (1995) Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature 378(6555):390–394

Genead R, Danielsson C, Andersson AB et al (2010) Islet-1 cells are cardiac progenitors present during the entire lifespan: from the embryonic stage to adulthood. Stem Cells Dev 19(10):1601–1615

Gessert S, Kuhl M (2010) The multiple phases and faces of wnt signaling during cardiac differentiation and development. Circ Res 107(2):186–199

Gnecchi M, He H, Noiseux N et al (2006) Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 20(6):661–669

Gomez-Marquez J, Franco del Amo F, Carpintero P et al (1996) High levels of mouse thymosin beta4 mRNA in differentiating P19 embryonic cells and during development of cardiovascular tissues. Biochim Biophys Acta 1306(2–3):187–193

Goumans MJ, de Boer TP, Smits AM et al (2007) TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. Stem Cell Res 1(2):138–149

Grego-Bessa J, Luna-Zurita L, del Monte G et al (2007) Notch signaling is essential for ventricular chamber development. Dev Cell 12(3):415–429

Gregory CJ, Eaves AC (1978) Three stages of erythropoietic progenitor cell differentiation distinguished by a number of physical and biologic properties. Blood 51(3):527–537

Haider H, Jiang S, Idris NM et al (2008) IGF-1-overexpressing mesenchymal stem cells accelerate bone marrow stem cell mobilization via paracrine activation of SDF-1alpha/CXCR4 signaling to promote myocardial repair. Circ Res 103(11):1300–1308

Hao J, Daleo MA, Murphy CK et al (2008) Dorsomorphin, a selective small molecule inhibitor of BMP signaling, promotes cardiomyogenesis in embryonic stem cells. PLoS One 3(8):e2904

Harada M, Qin Y, Takano H et al (2005) G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. Nat Med 11(3):305–311

Hare JM, Traverse JH, Henry TD et al (2009) A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J Am Coll Cardiol 54(24):2277–2286

Hasegawa H, Takano H, Iwanaga K et al (2006) Cardioprotective effects of granulocyte colony-stimulating factor in swine with chronic myocardial ischemia. J Am Coll Cardiol 47(4):842–849

Herdrich BJ, Danzer E, Davey MG et al (2010) Regenerative healing following foetal myocardial infarction. Eur J Cardiothorac Surg 38(6):691–698

Hinkel R, Bock-Marquette I, Hatzopoulos AK et al (2010) Thymosin beta4: a key factor for protective effects of eEPCs in acute and chronic ischemia. Ann N Y Acad Sci 1194:105–111

Hirata A, Minamino T, Asanuma H et al (2005) Erythropoietin just before reperfusion reduces both lethal arrhythmias and infarct size via the phosphatidylinositol-3 kinase-dependent pathway in Canine hearts. Cardiovasc Drugs Ther 19(1):33–40

Hsieh PC, Segers VF, Davis ME et al (2007) Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nat Med 13(8):970–974

Huang SM, Mishina YM, Liu S et al (2009) Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature 461(7264):614–620

Hynes B, Kumar AH, O'Sullivan J et al (2011) Potent endothelial progenitor cell-conditioned media-related anti-apoptotic, cardiotrophic, and pro-angiogenic effects post-myocardial infarction are mediated by insulin-like growth factor-1. Eur Heart J. doi: 10.1093/eurheartj/ehr435

Iekushi K, Seeger F, Assmus B et al (2012) Regulation of cardiac microRNAs by bone marrow mononuclear cell therapy in myocardial infarction. Circulation 125(14):1765–1773

Ince H, Petzsch M, Kleine HD et al (2005) Prevention of left ventricular remodeling with granulocyte colony-stimulating factor after acute myocardial infarction: final 1-year results of the Front-Integrated Revascularization and Stem Cell Liberation in Evolving Acute Myocardial Infarction by Granulocyte Colony-Stimulating Factor (FIRSTLINE-AMI) Trial. Circulation 112(9 Suppl):I73–I80

Iwasaki M, Adachi Y, Nishiue T et al (2005) Hepatocyte growth factor delivered by ultrasound-mediated destruction of microbubbles induces proliferation of cardiomyocytes and amelioration of left ventricular contractile function in Doxorubicin-induced cardiomyopathy. Stem Cells 23(10):1589–1597

Jiang ZS, Srisakuldee W, Soulet F et al (2004) Non-angiogenic FGF-2 protects the ischemic heart from injury, in the presence or absence of reperfusion. Cardiovasc Res 62(1):154–166

Jo JO, Kang YJ, Ock MS et al (2011) Thymosin beta4 expression in human tissues and in tumors using tissue microarrays. Appl Immunohistochem Mol Morphol 19(2):160–167

Johnston PV, Sasano T, Mills K (2009) Engraftment, differentiation, and functional benefits of autologous cardiosphere-derived cells in porcine ischemic cardiomyopathy. Circulation 120(12):1075–1083, 1077 p following 1083

Jopling C, Sleep E, Raya M et al (2010) Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. Nature 464(7288):606–609

Kajstura J, Leri A, Finato N et al (1998) Myocyte proliferation in end-stage cardiac failure in humans. Proc Natl Acad Sci USA 95(15):8801–8805

Kajstura J, Urbanek K, Perl S et al (2010) Cardiomyogenesis in the adult human heart. Circ Res 107(2):305–315

Kanashiro-Takeuchi RM, Schulman IH, Hare JM (2011) Pharmacologic and genetic strategies to enhance cell therapy for cardiac regeneration. J Mol Cell Cardiol 51(4):619–625

Kang HJ, Kim HS, Zhang SY et al (2004) Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. Lancet 363(9411):751–756

Karlsson O, Thor S, Norberg T et al (1990) Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. Nature 344(6269):879–882

Khattar P, Friedrich FW, Bonne G et al (2011) Distinction between two populations of islet-1-positive cells in hearts of different murine strains. Stem Cells Dev 20(6):1043–1052

Kikuchi K, Holdway JE, Werdich AA et al (2010) Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. Nature 464(7288):601–605

Komatsu N, H Nakauchi, A Miwa et al (1991) Establishment and characterization of a human leukemic cell line with megakaryocytic features: dependency on granulocyte-macrophage colony-stimulating factor, interleukin 3, or erythropoietin for growth and survival. Cancer Res 51: 341–348

Kraehenbuehl TP, Ferreira LS, Hayward AM et al (2011) Human embryonic stem cell-derived microvascular grafts for cardiac tissue preservation after myocardial infarction. Biomaterials 32(4):1102–1109

Kubin T, Poling J, Kostin S et al (2011) Oncostatin M is a major mediator of cardiomyocyte dedifferentiation and remodeling. Cell Stem Cell 9(5):420–432

Kuhn B, del Monte F, Hajjar RJ et al (2007) Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. Nat Med 13(8):962–969

Kukula K, Chojnowska L, Dabrowski M et al (2011) Intramyocardial plasmid-encoding human vascular endothelial growth factor A165/basic fibroblast growth factor therapy using percutaneous transcatheter approach in patients with refractory coronary artery disease (VIF-CAD). Am Heart J 161(3):581–589

Laflamme MA, Murry CE (2011) Heart regeneration. Nature 473(7347):326–335

Laugwitz KL, Moretti A, Lam J et al (2005) Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433(7026):647–653

Lee KF, Simon H, Chen H et al (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature 378(6555):394–398

Lepilina A, Coon AN, Kikuchi K et al (2006) A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. Cell 127(3):607–619

Li Q, Li B, Wang X et al (1997) Overexpression of insulin-like growth factor-1 in mice protects from myocyte death after infarction, attenuating ventricular dilation, wall stress, and cardiac hypertrophy. J Clin Invest 100(8):1991–1999

Li TS, Cheng K, Malliaras K et al (2012) Direct comparison of different stem cell types and subpopulations reveals superior paracrine potency and myocardial repair efficacy with cardiosphere-derived cells. J Am Coll Cardiol 59(10):942–953

Limana F, Capogrossi MC, Germani A (2011) The epicardium in cardiac repair: from the stem cell view. Pharmacol Ther 129(1):82–96

Lin FJ, Tsai MJ, Tsai SY (2007) Artery and vein formation: a tug of war between different forces. EMBO Rep 8(10):920–924

Lipsic E, van der Meer P, Voors AA et al (2006) A single bolus of a long-acting erythropoietin analogue darbepoetin alfa in patients with acute myocardial infarction: a randomized feasibility and safety study. Cardiovasc Drugs Ther 20(2):135–141

Loffredo FS, Steinhauser ML, Gannon J et al (2011) Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. Cell Stem Cell 8(4):389–398

Lorts A, Schwanekamp JA, Elrod JW et al (2009) Genetic manipulation of periostin expression in the heart does not affect myocyte content, cell cycle activity, or cardiac repair. Circ Res 104(1):e1–e7

Low TL, Hu SK, Goldstein AL (1981) Complete amino acid sequence of bovine thymosin beta 4: a thymic hormone that induces terminal deoxynucleotidyl transferase activity in thymocyte populations. Proc Natl Acad Sci USA 78(2):1162–1166

Makita T, Duncan SA, Sucov HM (2005) Retinoic acid, hypoxia, and GATA factors cooperatively control the onset of fetal liver erythropoietin expression and erythropoietic differentiation. Dev Biol 280(1):59–72

Makkar RR, Smith RR, Cheng K et al (2012) Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet 379(9819):895–904

Martin CM, Meeson AP, Robertson SM et al (2004) Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev Biol 265(1):262–275

McQuibban GA, Butler GS, Gong JH et al (2001) Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. J Biol Chem 276(47):43503–43508

Mercola M, Ruiz-Lozano P, Schneider MD (2011) Cardiac muscle regeneration: lessons from development. Genes Dev 25(4):299–309

Messina E, De Angelis L, Frati G et al (2004) Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 95(9):911–921

Meyer D, Birchmeier C (1995) Multiple essential functions of neuregulin in development. Nature 378(6555):386–390

Mirotsou M, Zhang Z, Deb A et al (2007) Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. Proc Natl Acad Sci USA 104(5):1643–1648

Mirotsou M, Jayawardena TM, Schmeckpeper J et al (2011) Paracrine mechanisms of stem cell reparative and regenerative actions in the heart. J Mol Cell Cardiol 50(2):280–289
Miyazaki M, Masaka T, Akiyama I et al (2004) Propagation of adult rat bone marrow-derived hepatocyte-like cells by serial passages in vitro. Cell Transplant 13(4):385–391
Moon C, Krawczyk M, Ahn D et al (2003) Erythropoietin reduces myocardial infarction and left ventricular functional decline after coronary artery ligation in rats. Proc Natl Acad Sci USA 100(20):11612–11617
Moretti A, Caron L, Nakano A et al (2006) Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 127(6):1151–1165
Murry CE, Soonpaa MH, Reinecke H et al (2004) Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428(6983):664–668
Nagasawa T, Hirota S, Tachibana K et al (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature 382(6592):635–638
Nakamura T, Nishizawa T, Hagiya M et al (1989) Molecular cloning and expression of human hepatocyte growth factor. Nature 342(6248):440–443
Nakamura T, Mizuno S, Matsumoto K et al (2000) Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. J Clin Invest 106(12):1511–1519
Oerlemans MI, Goumans MJ, van Middelaar B et al (2010) Active Wnt signaling in response to cardiac injury. Basic Res Cardiol 105(5):631–641
Oh H, Bradfute SB, Gallardo TD et al (2003) Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci USA 100(21):12313–12318
Oikonomopoulos A, Sereti KI, Conyers F et al (2011) Wnt signaling exerts an antiproliferative effect on adult cardiac progenitor cells through IGFBP3. Circ Res 109(12):1363–1374
Oka T, Xu J, Molkentin JD (2007) Re-employment of developmental transcription factors in adult heart disease. Semin Cell Dev Biol 18(1):117–131
Orlic D, Kajstura J, Chimenti S et al (2001a) Bone marrow cells regenerate infarcted myocardium. Nature 410(6829):701–705
Orlic D, Kajstura J, Chimenti S et al (2001b) Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA 98(18):10344–10349
Ott I, Schulz S, Mehilli J et al (2010) Erythropoietin in patients with acute ST-segment elevation myocardial infarction undergoing primary percutaneous coronary intervention: a randomized, double-blind trial. Circ Cardiovasc Interv 3(5):408–413
Padin-Iruegas ME, Misao Y, Davis ME et al (2009) Cardiac progenitor cells and biotinylated insulin-like growth factor-1 nanofibers improve endogenous and exogenous myocardial regeneration after infarction. Circulation 120(10):876–887
Pasumarthi KB, Nakajima H, Nakajima HO et al (2005) Targeted expression of cyclin D2 results in cardiomyocyte DNA synthesis and infarct regression in transgenic mice. Circ Res 96(1):110–118
Pearlman JD, Hibberd MG, Chuang ML et al (1995) Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. Nat Med 1(10):1085–1089
Peles E, Bacus SS, Koski RA et al (1992) Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. Cell 69(1):205–216
Perez-Pomares JM, Phelps A, Sedmerova M et al (2002) Experimental studies on the spatiotemporal expression of WT1 and RALDH2 in the embryonic avian heart: a model for the regulation of myocardial and valvuloseptal development by epicardially derived cells (EPDCs). Dev Biol 247(2):307–326
Pfister O, Mouquet F, Jain M et al (2005) CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. Circ Res 97(1):52–61
Pfister O, Oikonomopoulos A, Sereti KI et al (2010) Isolation of resident cardiac progenitor cells by Hoechst 33342 staining. Methods Mol Biol 660:53–63
Pittenger MF, Mackay AM, Beck SC et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284(5411):143–147

Porrello ER, Mahmoud AI, Simpson E et al (2011) Transient regenerative potential of the neonatal mouse heart. Science 331(6020):1078–1080
Poss KD, Wilson LG, Keating MT (2002) Heart regeneration in zebrafish. Science 298(5601):2188–2190
Psaltis PJ, Paton S, See F et al (2010) Enrichment for STRO-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. J Cell Physiol 223(2):530–540
Rasmussen TL, Raveendran G, Zhang J et al (2011) Getting to the heart of myocardial stem cells and cell therapy. Circulation 123(16):1771–1779
Ratajska A, Czarnowska E, Ciszek B (2008) Embryonic development of the proepicardium and coronary vessels. Int J Dev Biol 52(2–3):229–236
Reber L, Da Silva CA, Frossard N (2006) Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. Eur J Pharmacol 533(1–3):327–340
Reeves I, Abribat T, Laramee P et al (2000) Age-related serum levels of insulin-like growth factor-I, -II and IGF-binding protein-3 following myocardial infarction. Growth Horm IGF Res 10(2):78–84
Ripa RS, Jorgensen E, Wang Y et al (2006) Stem cell mobilization induced by subcutaneous granulocyte-colony stimulating factor to improve cardiac regeneration after acute ST-elevation myocardial infarction: result of the double-blind, randomized, placebo-controlled stem cells in myocardial infarction (STEMMI) trial. Circulation 113(16):1983–1992
Rota M, Padin-Iruegas ME, Misao Y et al (2008) Local activation or implantation of cardiac progenitor cells rescues scarred infarcted myocardium improving cardiac function. Circ Res 103(1):107–116
Russell JL, Goetsch SC, Gaiano NR et al (2011) A dynamic notch injury response activates epicardium and contributes to fibrosis repair. Circ Res 108(1):51–59
Russell JL, Goetsch SC, Aguilar HR et al (2012) Targeting native adult heart progenitors with cardiogenic small molecules. ACS Chem Biol 7(6):1067–1076
Sadek H, Hannack B, Choe E et al (2008) Cardiogenic small molecules that enhance myocardial repair by stem cells. Proc Natl Acad Sci USA 105(16):6063–6068
Safer D, Elzinga M, Nachmias VT (1991) Thymosin beta 4 and Fx, an actin-sequestering peptide, are indistinguishable. J Biol Chem 266(7):4029–4032
Salmonson T, Danielson BG, Wikstrom B (1990) The pharmacokinetics of recombinant human erythropoietin after intravenous and subcutaneous administration to healthy subjects. Br J Clin Pharmacol 29(6):709–713
Saraswati S, Alfaro MP, Thorne CA et al (2010) Pyrvinium, a potent small molecule Wnt inhibitor, promotes wound repair and post-MI cardiac remodeling. PLoS One 5(11):e15521
Schultheiss TM, Burch JB, Lassar AB (1997) A role for bone morphogenetic proteins in the induction of cardiac myogenesis. Genes Dev 11(4):451–462
Scott RC, Rosano JM, Ivanov Z et al (2009) Targeting VEGF-encapsulated immunoliposomes to MI heart improves vascularity and cardiac function. FASEB J 23(10):3361–3367
Segers VF, Lee RT (2008) Stem-cell therapy for cardiac disease. Nature 451(7181):937–942
Segers VF, Tokunou T, Higgins LJ et al (2007) Local delivery of protease-resistant stromal cell derived factor-1 for stem cell recruitment after myocardial infarction. Circulation 116(15):1683–1692
Smart N, Risebro CA, Melville AA et al (2007) Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. Nature 445(7124):177–182
Smart N, Dube KN, Riley PR (2010) Identification of Thymosin beta4 as an effector of Hand1-mediated vascular development. Nat Commun 1:46
Smart N, Bollini S, Dube KN et al (2011) De novo cardiomyocytes from within the activated adult heart after injury. Nature 474(7353):640–644
Smits AM, van Vliet P, Hassink RJ et al (2005) The role of stem cells in cardiac regeneration. J Cell Mol Med 9(1):25–36
Soonpaa MH, Field LJ (1998) Survey of studies examining mammalian cardiomyocyte DNA synthesis. Circ Res 83(1):15–26
Soonpaa MH, Kim KK, Pajak L et al (1996) Cardiomyocyte DNA synthesis and binucleation during murine development. Am J Physiol Heart Circ Physiol 271(5):H2183–H2189

Stanton LW, Garrard LJ, Damm D et al (2000) Altered patterns of gene expression in response to myocardial infarction. Circ Res 86(9):939–945
Steinhauser ML, Lee RT (2011) Regeneration of the heart. EMBO Mol Med 3(12):701–712
Sugano Y, Anzai T, Yoshikawa T et al (2005) Granulocyte colony-stimulating factor attenuates early ventricular expansion after experimental myocardial infarction. Cardiovasc Res 65(2):446–456
Sundararaman S, Miller TJ, Pastore JM et al (2011) Plasmid-based transient human stromal cell-derived factor-1 gene transfer improves cardiac function in chronic heart failure. Gene Ther 18(9):867–873
Sussman MA, Murry CE (2008) Bones of contention: marrow-derived cells in myocardial regeneration. J Mol Cell Cardiol 44(6):950–953
Suzuki N, Ohneda O, Takahashi S et al (2002) Erythroid-specific expression of the erythropoietin receptor rescued its null mutant mice from lethality. Blood 100(7):2279–2288
Syed RS, Reid SW, Li C et al (1998) Efficiency of signalling through cytokine receptors depends critically on receptor orientation. Nature 395(6701):511–516
Tada H, Kagaya Y, Takeda M et al (2006) Endogenous erythropoietin system in non-hematopoietic lineage cells plays a protective role in myocardial ischemia/reperfusion. Cardiovasc Res 71(3):466–477
Tallini YN, Greene KS, Craven M et al (2009) c-kit expression identifies cardiovascular precursors in the neonatal heart. Proc Natl Acad Sci USA 106(6):1808–1813
Tang J, Wang J, Guo L et al (2010) Mesenchymal stem cells modified with stromal cell-derived factor 1 alpha improve cardiac remodeling via paracrine activation of hepatocyte growth factor in a rat model of myocardial infarction. Mol Cells 29(1):9–19
Tillmanns J, Rota M, Hosoda T et al (2008) Formation of large coronary arteries by cardiac progenitor cells. Proc Natl Acad Sci USA 105(5):1668–1673
Tomita Y, Matsumura K, Wakamatsu Y et al (2005) Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart. J Cell Biol 170(7):1135–1146
Torella D, Rota M, Nurzynska D et al (2004) Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. Circ Res 94(4):514–524
Urbanek K, Rota M, Cascapera S et al (2005) Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. Circ Res. 01.RES.0000183733.0000153101.0000183711
Urbich C, Aicher A, Heeschen C et al (2005) Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 39(5):733–742
van Vliet P, Roccio M, Smits AM et al (2008) Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. Neth Heart J 16(5):163–169
Vantler M, Karikkineth BC, Naito H et al (2010) PDGF-BB protects cardiomyocytes from apoptosis and improves contractile function of engineered heart tissue. J Mol Cell Cardiol 48(6):1316–1323
Wang Y, Ahmad N, Wani MA et al (2004) Hepatocyte growth factor prevents ventricular remodeling and dysfunction in mice via Akt pathway and angiogenesis. J Mol Cell Cardiol 37(5):1041–1052
Wang H, Hao J, Hong CC (2011) Cardiac induction of embryonic stem cells by a small molecule inhibitor of Wnt/beta-catenin signaling. ACS Chem Biol 6(2):192–197
Watanabe E, Smith DM, Sun J et al (1998) Effect of basic fibroblast growth factor on angiogenesis in the infarcted porcine heart. Basic Res Cardiol 93(1):30–37
Watanabe Y, Kokubo H, Miyagawa-Tomita S et al (2006) Activation of Notch1 signaling in cardiogenic mesoderm induces abnormal heart morphogenesis in mouse. Development 133(9):1625–1634
Wei HM, Wong P, Hsu LF et al (2009) Human bone marrow-derived adult stem cells for post-myocardial infarction cardiac repair: current status and future directions. Singapore Med J 50(10):935–942
Weinberger F, Mehrkens D, Friedrich FW et al (2012) Localization of Islet-1-positive cells in the healthy and infarcted adult murine heart. Circ Res 110(10):1303–1310

Winter EM, Gittenberger-de Groot AC (2007) Epicardium-derived cells in cardiogenesis and cardiac regeneration. Cell Mol Life Sci 64(6):692–703

Xu B, Dong G-h, Liu H et al (2005) Recombinant human erythropoietin pretreatment attenuates myocardial infarct size: a possible mechanism involves heat shock protein 70 and attenuation of nuclear factor-kappaB. Ann Clin Lab Sci 35(2):161–168

Xu Y, Li X, Liu X et al (2010) Neuregulin-1/ErbB signaling and chronic heart failure. Adv Pharmacol 59:31–51

Yanagisawa-Miwa A, Uchida Y, Nakamura F et al (1992) Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. Science 257(5075):1401–1403

Yasuda H, Galli SJ, Geissler EN (1993) Cloning and functional analysis of the mouse c-kit promoter. Biochem Biophys Res Commun 191(3):893–901

Ye L, Du X, Xia J et al (2005) Effects of rHu-EPO on myocyte apoptosis and cardiac function following acute myocardial infarction in rats. J Huazhong Univ Sci Technolog Med Sci 25(1):55–58

Yoon YS, Wecker A, Heyd L et al (2005) Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. J Clin Invest 115(2):326–338

Yu PB, Hong CC, Sachidanandan C et al (2008) Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 4(1):33–41

Yuasa S, Itabashi Y, Koshimizu U et al (2005) Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. Nat Biotechnol 23(5):607–611

Zaruba MM, Theiss HD, Vallaster M et al (2009) Synergy between CD26/DPP-IV inhibition and G-CSF improves cardiac function after acute myocardial infarction. Cell Stem Cell 4(4):313–323

Zaruba MM, Soonpaa M, Reuter S et al (2010) Cardiomyogenic potential of C-kit(+)-expressing cells derived from neonatal and adult mouse hearts. Circulation 121(18):1992–2000

Zeisberg EM, Tarnavski O, Zeisberg M et al (2007) Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 13(8):952–961

Zelarayan LC, Noack C, Sekkali B et al (2008) Beta-Catenin downregulation attenuates ischemic cardiac remodeling through enhanced resident precursor cell differentiation. Proc Natl Acad Sci USA 105(50):19762–19767

Zhao YY, Sawyer DR, Baliga RR et al (1998) Neuregulins promote survival and growth of cardiac myocytes Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. J Biol Chem 273(17):10261–10269

Zhou S, Schuetz JD, Bunting KD et al (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 7(9):1028–1034

Zhou B, Ma Q, Rajagopal S (2008) Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature 454(7200):109–113

Zhou B, Honor LB, Ma Q et al (2012) Thymosin beta 4 treatment after myocardial infarction does not reprogram epicardial cells into cardiomyocytes. J Mol Cell Cardiol 52(1):43–47

Zohlnhofer D, Ott I, Mehilli J et al (2006) Stem cell mobilization by granulocyte colony-stimulating factor in patients with acute myocardial infarction: a randomized controlled trial. JAMA 295(9):1003–1010

Zou YR, Kottmann AH, Kuroda M et al (1998) Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393(6685):595–599

Chapter 26
Blood

Michael Schmitt and Mathias Freund

Abstract The transplantation of stem cells from the bone marrow, peripheral blood or cord blood has become a clinical procedure since the 1980s and is now annually performed in ten thousands of patients in the autologous and allogeneic setting world-wide. Refinement of human leukocyte antigen typing as well as recent advances in immunosuppression, anti-infective prophylaxis and therapy as well in supportive care have much improved the outcome of patients with leukemia and lymphoma, aplastic anemia, as well as hereditary diseases of the hematopoietic system. This is still an experimental therapy and patient subgroups that profit most from hematopoietic stem cell transplantation need to be defined. Consideration and classification of co-morbidity indices as well as cytogenetic risk factors are pivotal for making decisions on transplantation modalities. Modern conditioning regimens allow balancing of allo-effects against malignant cells versus normal tissue even in elderly patients. Recent innovations in cellular therapy combine allogeneic stem cell transplantation with genetically engineered or specifically selected T cells and potentially natural killer (NK) cells. Depletion of regulatory T cells and vaccination after allogeneic stem cell transplantation constitute further approaches to improve the long-term outcome of transplanted patients.

M. Schmitt, M.D., Ph.D., MHBA (✉)
Cellular Immunotherapy, University Clinic Heidelberg,
Im Neuenheimer Feld 410, 69120 Heidelberg, Germany
e-mail: Michael.Schmitt@med.uni-heidelberg.de

M. Freund, M.D., Ph.D.
3rd Medical Department, Hematology, Oncology, Palliative Medicine,
Center of Medicine, Rostock University Hospitals,
Ernst-Heydemann-Str. 6, D-18055 Rostock, Germany

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_26,

26.1 Blood: A Long Way from Replacement to Regeneration

Have you ever asked yourself why the red lights at the crossing are red? It is the color of blood that warns for danger. Blood has ever fascinated man. It is the topic of myths and rites. The shedding of blood initiates deeply rooted fears.

The first documented attempt to replace this essence of life and to cure deadly disease has been performed in Paris on the 15th of June 1667 by Jean-Baptiste Denis. Blood from a lamb was transfused to a 15 year old boy who survived. More than 300 years later Landsteiner discovered the A, B, O blood group system. He received the Noble Prize for his research in 1930. He laid the fundament for the first successful blood transfusion 1907 in New York by Reuben. 1915 it became possible to conserve blood for transfusion by the addition of citrate. Blood group testing was refined with the discovery of the N, M, and P system and finally in 1939 by the discovery of the Rh system. Blood transfusions increasingly became an essential treatment on the battle fields of the Second World War and later in the Korean War.

As spontaneous recovery from blood loss is evident in healthy subjects and as there are blood diseases in which lifelong blood replacement would be needed the need to advance from replacement to regeneration was obvious. An era of intensive research on bone marrow and its stem cells began in the 1950s.

It was discovered that the infusion of spleen cells promoted blood regeneration and led to survival of supralethal total body irradiation (Barnes and Loutin 1953; Ford et al. 1956; Nowell et al. 1956; Vos et al. 1956). However in 1957 first attempts of clinical bone marrow transplantation in man were unsuccessful in the majority of cases. The reasons were allograft failure and progressive disease (Thomas et al. 1957). Two years later Thomas reported the first successful allogeneic bone marrow transplants from identical twins in two patients with acute lymphoblastic leukemia.

It is remarkable that this program had been started by E.D. Thomas in 1955. It was years before stem cell assays were developed by Donald Metcalf (Bradley and Metcalf 1966) and Leo Sachs (Ginsburg and Sachs 1963). The theoretical and experimental basis of stem cell transplantation had been left behind by clinical application.

Although many human blood stem cell transplantations were carried out between 1958 and 1968, the outcome had not been encouraging. Out of 203 patients transplanted in these times, 125 experienced graft failure, 49 developed lethal graft-versus-host disease (GvHD), and only 11 achieved long-term engraftment. Only three patients were alive when Bortin reported these results in 1970 (Bortin 1970). Many researches left the field and some voices declared hematopoietic stem cell transplantation as dead.

Those who were not discouraged returned to the laboratory and animal models. After progress in the understanding of the HLA-system and the development of GvHD prophylaxis by immunosuppression transplantation went back to the clinics. The Seattle group realized that patients with far advanced malignant disease and poor general status had a dismal outcome in contrast too patients in the earlier stages (Thomas et al. 1975). It was an enormous venture at that time to transform this finding into a clinical consequence: to recommend the dangerous procedure to

patients in remission, to patients with non malignant or low malignant but long-term dismal disease, and children.

This courage of the early clinical researches and the following developments in high resolution HLA typing, worldwide donor programs, the improvement of the conditioning regimen and supportive care have established hematopoietic stem cell transplantation as the first true regenerative therapy. We can learn from that development for other areas of regenerative medicine.

Yet the field of hematopoietic stem cell transplantation is still dynamic. New indications emerge as the procedure is improved. Other indications vanish as their conservative treatment advances. It is the venue of this chapter to give an insight into the actual status of this treatment option.

26.2 Hematopoietic Stem Cells

26.2.1 Basic Properties

Hematopoietic stem cells have the capacity to self-renew and to differentiate in all mature blood lineages (Fig. 26.1). They have been first identified in the mid 1950s by their capability to rescue lethally irradiated mice by reconstituting the entire repertoire of hematopoietic cells. Hematopoietic stem cells are scarce with a frequency of 1:10,000 to 1,000,000 bone marrow cells. Without stress the majority of stem cells rest in a quiescent state while only a small fraction enters the cell cycle and proliferates to give rise to differentiated progenitors. During infections, acute bleeding, or chemotherapy a large fraction of the stem cells may proliferate.

The regenerative capacity of the stem cells has its evidence in the fact that despite the short lived nature of blood cells a continuous supply of these cells is given even in very long living persons without clinical signs of insufficiency. The self renewal potential of the hematopoietic stem cells is associated with the activity of telomerase. The telomeres at the end of the chromosomes shorten during cell division. This process is reduced by telomerase, a reverse transcriptase which synthesizes new telomeric DNA (Morrison et al. 1996). Telomere shortening is associated with cell cycle arrest, replicative senescence and chromosomal instability. It might be an inhibitory mechanism against the evolution of malignant cell clones.

Despite the activity of telomerase in hematopoietic stem cells, their replication capacity is limited. Serial stem cell transplantations in mice can be done with minimal stem cell numbers for five to seven times until hematopoietic insufficiency occurs (Harrison and Astle 1982). On the other hand it should be noted that transplantation is a severe stress for stem cells. The regenerative potential of stem cells under normal conditions is enormous. It has been concluded from these mouse experiments that hematopoietic stem cells should be able to function normally through at least 15–50 life spans. Therefore hematopoietic insufficiency should not be expected in even very old subjects.

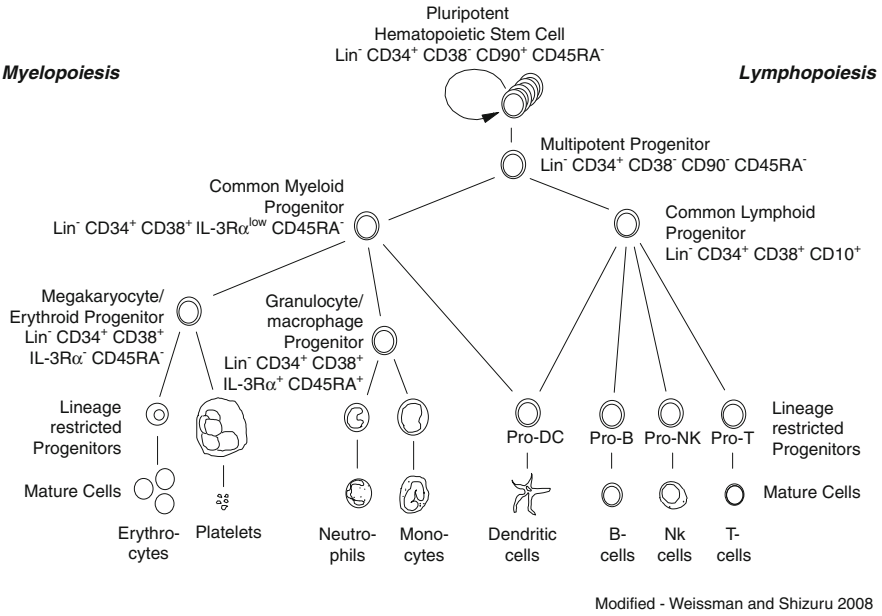


Fig. 26.1 Pedigree of the normal hematopoiesis through the influence of cytokines and growth factors like interleukin 3 (IL-3), stem cells differentiate into mature cells of the peripheral blood. Partially differentiated progenitor cells are characterized by surface markers, so called "clusters of differentiation" (CD) as indicated

26.2.2 Characterization of Hematopoietic Stem Cells

The study of hematopoietic stem cells is difficult because of their low frequency in the bone marrow. Specific markers or tests for a definitive identification of stem cells are lacking. So in most instances methods have to be combined for the characterisation of stem cells.

26.2.2.1 Surface Markers

In mice hematopoietic stem cells are fairly well characterized by surface markers. A single murine bone marrow cell which is CD34-/lo, CD117+ Sca1+ (stem cell antigen) and negative for lineage-specific antigens is capable of self renewal and multi-lineage differentiation when transplanted into a recipient mouse (Ema et al. 2000). In humans the phenotypic properties of hematopoietic stem cells are far less well defined.

26.2.2.2 Stem Cells and the Concept of "Niches"

There exists a more than 30 year old concept that the number and behavior of hematopoietic stem cells (HSCs) is regulated by physically discrete locations within the bone marrow for which the French term "niches" was coined. Despite the fact that the precise

identities of the niche cells are not yet well defined and controversial, there is an increasing body of evidence that HSCs are retained within the niches by specific adhesion molecules and chemokine gradients (Papayannopoulou and Scadden 2008). By these interactions, HSCs can be assured that they receive appropriate supportive signals that allow them to retain their stem cell identity. In contrast to this concept, there are data suggesting that recipient bone marrow can be readily displaced by transplanted marrow in an efficient and linear dose-dependent manner, even in the absence of conditioning (Colvin et al. 2004). These authors have described a model where HSCs do not reside locked into fixed positions in the bone marrow, but instead they would receive their regulatory signals through limiting quantities of freely diffusible factors.

Work by Irvin Weissman and co-workers (Bhattacharya et al. 2009; Czechowicz et al. 2007) clearly demonstrated that a certain degree of HSC replacement occurs even in the absence of conditioning. Recent studies could demonstrate that egress of HSCs can be stimulated pharmacologically through administration of plerixafor (AMD3100), an inhibitor of CXCR4. This resulted in the clearance of niches from HSCs. As HSCs and progenitors have been demonstrated to circulate under physiological conditions, a steady-state HSC egress from niches might also allow the engraftment of donor HSCs. One to five percent of HSCs in the murine model enter into the circulating pool every day.

Weissman's group fed their mice with bromodeoxyuridine and found out that HSCs in the circulating pool incorporate the dye at the same rate as bone marrow HSCs (Fig. 26.2). This suggests that HSCs egress from the bone marrow to the blood without cell division and can leave behind them vacant HSC niches (Bhattacharya et al. 2009).

26.2.3 Stem Cell Sources

Initially, bone marrow was obtained from healthy HLA-matched sibling donors of the patients. Donors were subjected to intubation and anesthesia. In a prostate position bone marrow was aspirated from the upper posterior iliac crest and transferred into a transfusion bag. After stem cell counting and microbiological and virological evaluation of the bone marrow blood preparation the stem cell containing bone marrow is transfused into the patients by the way of a central venous catheter.

Bone marrow aspiration under sterile conditions in the operation theater is costly and cumbersome. Moreover, stem cells mobilized from the bone marrow niches into the peripheral blood by subcutaneous administration of granulocyte-colony stimulation factor (G-CSF) or plerixafor to the donor will result in a 1 week earlier engraftment when compared with bone marrow. Therefore most of the stem cell preparations given nowadays to adult patients are peripheral blood stem cells collected by leukapheresis (Fig. 26.3). Through magnetic cell separation using anti-CD34 monoclonal antibodies labeled with magnetic beads (Fig. 26.4) a highly purified fraction of CD34+ stem cells can be prepared. Only for younger patients with e.g. aplastic anemia (see below), bone marrow stem cells are preferred due to the better reconstitution of the bone marrow with all its components (Schrezenmeier et al. 2007).

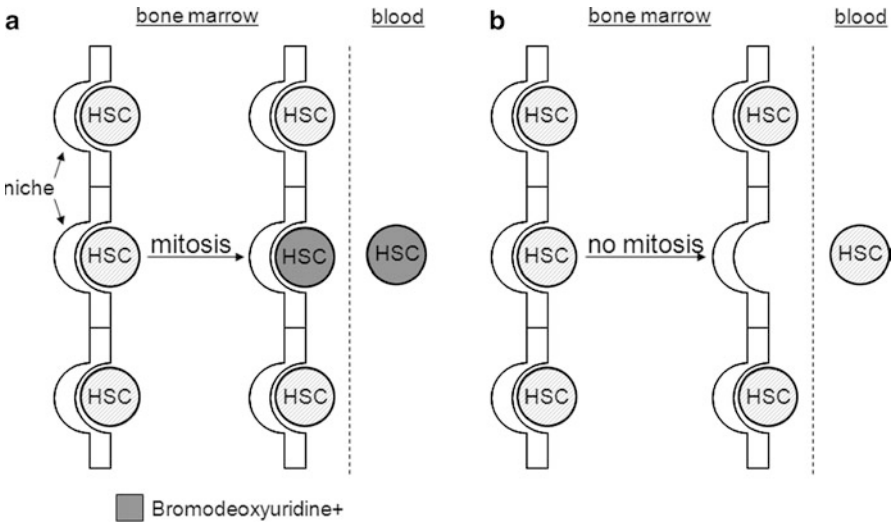


Fig. 26.2 HSC egress is either division dependent or independent (From Bhattacharya et al. 2009). HSCs can either undergo an extrinsically asymmetric division, in which one daughter cell is positioned away from a supportive niche and can thus intravasate to the blood (**a**) or can exit the supportive niche in the absence of cellular division (**b**). In the former model, all HSCs in the blood would be expected to have incorporated BrdU (*gray shaded cells*) after an appropriate feeding period, while the latter model would predict similar low BrdU incorporation rates between bone marrow and blood HSCs

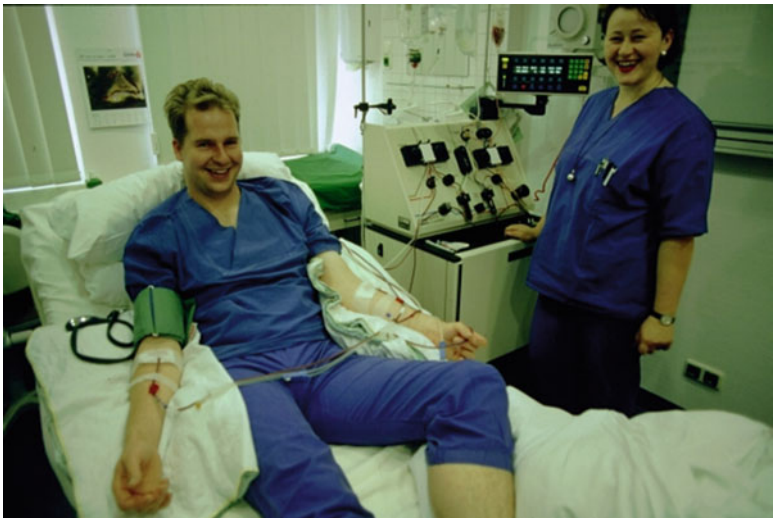


Fig. 26.3 Stem cell donor during leukapheresis. A leukapheresis device is put into a circuit between the *left* and the *right* cubital veins. It separates white blood cells containing stem cells by a density gradient method

Fig. 26.4 Purification of stem cells through a magnetic cell separation device (CliniMACS® by Miltenyi, Bergisch Gladbach/Teterow, Germany). Cells (in the *red bag* above the device) are labeled by monoclonal antibodies (moAbs) against the stem cell marker CD34. These moAbs are coupled with magnetic beads. The beads stick to a magnet during the 1st run of a buffer (the bag with a clear solution on the *right side*) though the column. When the column is detached from the magnet, a second eluate containing the marker-positive (stem) cells can be obtained

A novel source for hematopoietic stem cell is cord blood. Its application has been initiated by Eliane Gluckman at the Hôpital Saint Louis, Sorbonne VII Paris (Czechowicz et al. 2007) and has been practiced in more than 4,000 patients in Japan (Kodera 2008). Hitherto, the transplantation of cord blood derived stem cells is restricted by the number of stem cells from this source when put into relation with the average body weight of a European patient undergoing HSCT. Application of several cord blood preparations as "dual" or even "triple" cord blood transplantation gets into practice (Arachchillage et al. 2010). While the extended time of engraftment make the patient even more prone to opportunistic infections, cord blood stem cells do obviously not have to be HLA-identical Mismatches cause less GvHD in the cord blood setting than in the setting of PBSCT or bone marrow transplantation (Barker et al. 2009).

26.2.4 Stem Cell Doses for Transplantation

Different sources of stem cells like bone marrow, peripheral blood and cord blood can yield different amounts of stem cells with various pros and cons (Table 26.1).

For autologous transplantation one would like to administer 2×10^6/kg body weight (BW) of the recipient. In the case of allogeneic HSCT the desirable CD34+

Table 26.1 Stem cell sources and stem cell doses

Source	Pro's/Con's	CD34+ stem cell count
Bone marrow	Aspiration requires general anesthesia	Median of 2.8×10^6/kg body weight (BW)
Peripheral blood	Easy collection, but G-CSF side effects	Median of 7.0×10^6/kg BW
Cord blood	Easy, immediately available, partial HLA mismatches acceptable	Median of 0.2×10^6/kg BW

stem cell count would be at least $>2.5 \times 10^6$/kg BW, better $>5.0 \times 10^6$/kg BW for peripheral blood stem cells, and at least $>1.0 \times 10^6$/kg BW, better $>2.5 \times 10^6$/kg BW for bone marrow blood stem cells.

26.3 Principles of Hematopoietic Transplantation for Regeneration in Blood Diseases

Hematopoietic stem cell transplantation can be performed in two principally different situations: (1) Stem cells can be harvested in a patient with malignant disease and can be used to induce and/or accelerate hematopoietic regeneration after myelosuppressive or myeloablative treatment procedures. (2) Stem cells from a healthy volunteer donor can be transplanted for hematopoietic recovery of patients with non-malignant and malignant blood disorders.

26.3.1 Autologous Transplantation

The rationale in the autologous setting is to deliver as intensive cytotoxic treatment to the patient as possible. The basis of this concept is the finding that in a certain dose range of irradiation or cytotoxic treatment, the effect on the tumor increases in a steep linear relationship (Fig. 26.5). However this dose range is not equal in all tumors and with increasing doses there is increasing damage to hematopoiesis and organs. As organ damage may occur later in some agents an autologous transplantation of hematopoietic stem cells might open a therapeutic window for dose intensification.

This concept (Fig. 26.6) has been proven most convincingly in lymphomas, Hodgkin's disease and multiple myeloma. Attempts to apply high dose chemotherapy in other diseases as sarcomas or some other solid tumors have not been as successful, probably due to the fact that chemotherapies active in these diseases are very toxic to the organs so that the window opened by autologous hematopoietic transplantation is small or non-existing. Although autologous transplantation is given even in disseminated hematologic disease as the acute leukemias, this

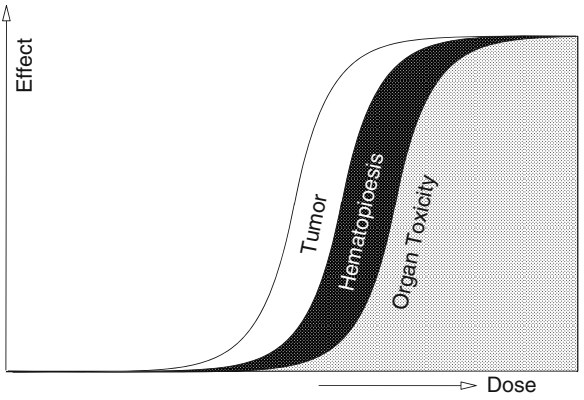


Fig. 26.5 Dose of cytotoxic treatment and toxic effects on tumor, hematopoiesis and organs. The higher the dose, the myelotoxicity or even organ toxicity

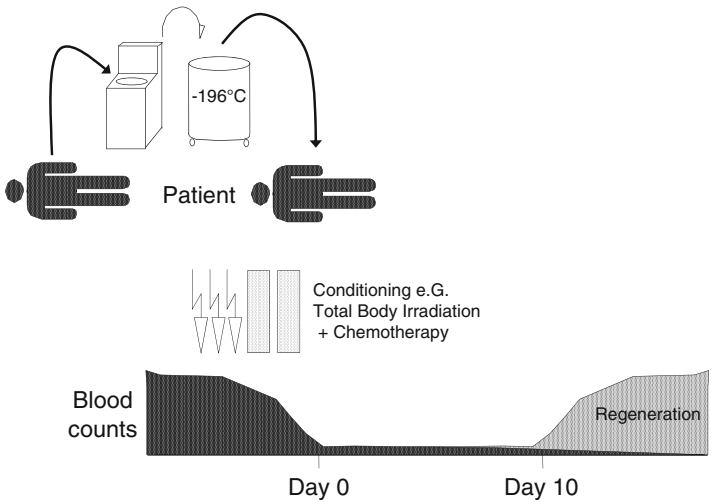


Fig. 26.6 Schema of autologous transplantation. Stem cells are collected from a hematological patient by leukapheresis after mobilization with e.g. cyclophosphamide and granulocyte-colony stimulating factor (G-CSF). Stem cells are stored in liquid nitrogen. The patient undergoes a conditioning regimen with e.g. total body irradiation (TBI) and cyclophosphamide. Thereafter the autologous stem cells are given back to the patient. The blood counts drop under a conditioning regimen of TBI and chemotherapy. Regeneration of hematopoiesis starts by day 10 after transplantation

treatment principle is not convincing in these entities as potentially tumor stem cells are also re-infused with the graft. Purification of grafts by chemical of immunologic methods have not yielded better results and are hampered by side effect e.g. immunosuppression by T-cell depletion in CD34+ selected grafts.

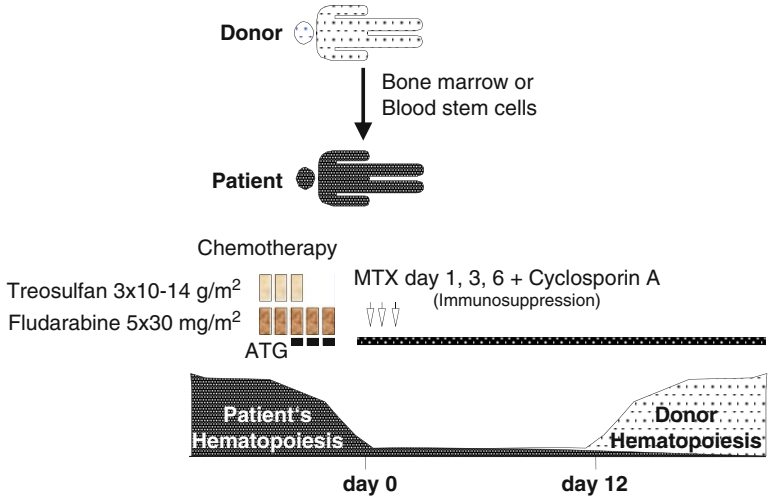


Fig. 26.7 Schema of allogeneic transplantation with the treosulfan-fludarabine conditioning regimen. After a conditioning with a treosulfane and fludarabine containing chemotherapy regimen the patient obtains stem cells from the bone marrow or peripheral blood of a healthy donor. To avoid graft rejection immunosuppression with e.g. methotrexate (MTX) or cyclosporine A is administered. As early as by day 12 reconstitution of the hematopoietic system is effective

26.3.2 Allogeneic Transplantation

The basic concept of allogeneic transplantation is to replace malignant or deficient hematopoiesis by transplantation from another individual (Fig. 26.7). Consequently allogeneic transplantation has been first applied in irradiation injuries and aplastic anemia.

As immunocompetent cells are normally transferred with the graft and as minor immunologic disparancies might lead to a graft-versus-host reaction concomitant immunosuppressive prophylaxis has to be given in the allogeneic setting. However in contrast to organ transplantation the immunosuppression can be omitted in most patients after immunologic reconstitution.

Allogeneic transplantation is widely applied in children and adult patients with genetic diseases, immunodeficiency syndromes, aplastic anemia, leukemias and lymphomas.

26.4 Diagnostics and Indications of "*Blood*" Regenerative Therapies

Histocompatibility is a basic prerequisite for allogeneic transplantation. Future concepts of allogeneic transplantation might overcome this principle to some extent. Another very important limitation for the application of allogeneic transplantation

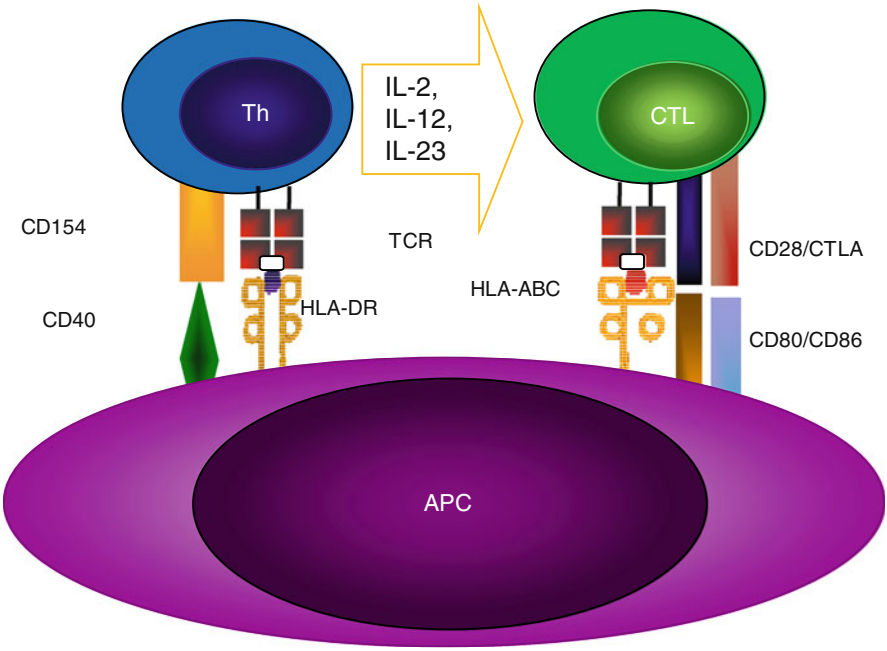


Fig. 26.8 Recognition of antigen epitopes by CD4+ and CD8+ T lymphocytes. Peptides derived from antigens are presented on HLA-molecules class I and II. A second signal is effective by binding of costimulatory molecules with their ligands. Interaction of T helper cells and cytotoxic T cells occurs through interleukines. *CTL* CD8+ cytotoxic T lymphocytes, *Th* CD4+ T helper cells, *TCR* T cell receptor, *IL* Interleukin, *CD* clusters of differentiation, *DC40, CD80, CD86* Co-stimulatory moleculars, *HLA* human leukocytes antigen

is the presence of comorbidities of the patients which become more prevalent with increasing age. A third overwhelming important factor for the outcome after autologous and allogeneic transplantation is the disease status and risk group. The latter is discussed later.

26.4.1 HLA Compatibility

The human leukocyte antigen (HLA) system is the human equivalent of the major histocompatibility complex. T cell recognition of peptide epitopes derived from functional or structural proteins is essential for the engraftment or rejection of the transplant and directly linked to GVHD and GVL reactions. Two classes of HLA molecules can be distinguished: HLA-A,-B and -Cw are class I molecules with three alpha subunits and a beta-2 microglobulin molecule, while class II molecules such as HLA-DR,-DQ and -DP are composed of two alpha and two beta subunits (see Fig. 26.8) (Klein and Sato 2000a, b). In their groove, HLA-ABC molecules present 9–11 amino acid residues long peptides to the T cell receptor of CD8+ T

Table 26.2 Difference in HLA frequencies in different ethnical groups

	African Americans 447	USA Caucasians 246	Japanese 1023	No. American Amerindians 51	Amerindians Chile-Colombia 72
A1	5.3	**16.9**	0.7	4.9	6.9
A2	16.7	**28.3**	**24.4**	**25.5**	**37.1**
A24	**4.7**	9.6	35.1	19.6	**33.7**
A26	**1.6**	**3.9**	**10.9**	**2**	**0.7**
B35	7.7	8.5	8.1	**18.6**	**29.8**
B39	0.8	1.8	4.5	**17.4**	**17.9**
Cw7	**15.6**	**21.5**	**15.3**	**30.7**	**26.9**
DR3	**14.7**	**10.1**	0.2		2.3
DR7	9.5	**15.1**	0.4		0.8

Data from the 11th International Histocompatibility Workshop (1991)

lymphocytes while class II HLA-D molecules present 15-20mer peptides to CD4+ T lymphocytes (Rammensee et al. 1993).

All HLA molecules are encoded by the short arm of chromosome 6. The DNA encoding class I and II molecules is separated by the DNA encoding hydroxylase 21 (C21) and tumor necrosis factor (TNF) (Trowsdale and Campbell 1988). The definition of ten parameters, i.e. the maternal and paternal HLA-**A**,-B,-C, DR and –DQ in both donor and recipient of an allograft is of crucial importance for stem cell transplantation, and to a lesser extent for solid organ transplantation (SOT). A so called two-digit typing is possible through serological assays employing anti-sera of defined specificity. Antigen-subtypes are called "splits". HLA subtypes are classified in International Workshops; "w" in the classification of HLA subtypes stands for "workshop" which means that subtypes will be further classified in future workshops, e.g. HLA-Cw4 etc. The HLA subtypes are differentially expressed in various ethnic groups such as Caucasians, African Black, Hispanics, Han Chinese, and Japanese (Table 26.2).

Minor histocompatibility antigens (miHAgs) such as HA-1 (den Haan et al. 1998) can both elict GVHD (Mutis et al. 1999) but can also contribute to the recognition of leukemic blast i.e. to the GVL (Goulmy et al. 1996) effect. Moreover natural killer cells, their surface molecules and killer cell inhibitory (KIR) molecules contribute GVHD and GVL, and are of pivotal importance for allogeneic stem cell transplantations in the haploidentical setting (Moretta et al. 2009).

26.4.2 EBMT Comorbidity Score

Previously, Alois Gratwohl and his working party established the European Group for Blood and Marrow Transplantation (EBMT) risk score for allogeneic transplantation in chronic myeloid leukemia (CML). Recently, it was questioned whether this score could be also used to predict outcome after allogeneic hematopoietic stem cell transplantation (HSCT) for hematological disease in general (Fig. 26.9). Age of

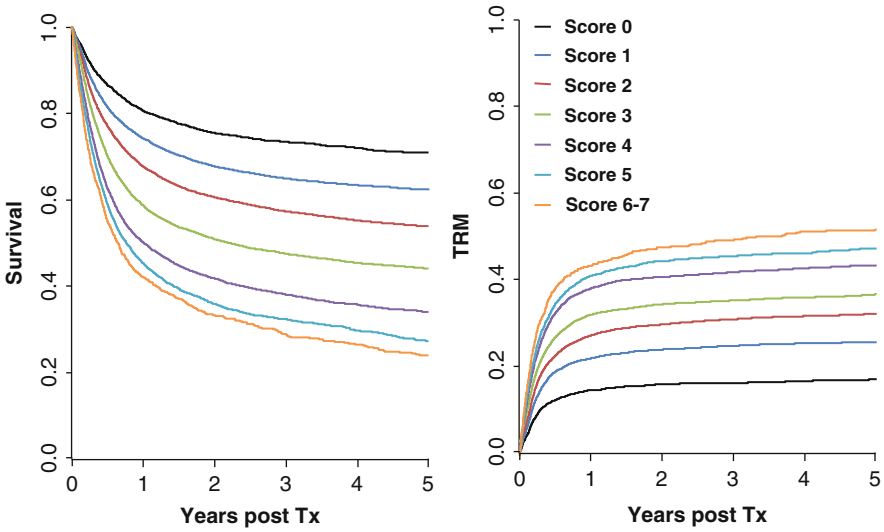


Fig. 26.9 The EBMT risk score. One can stratify patients into several risk groups and employs only five parameters like age of the patient, disease stage, time interval from diagnosis to transplant, donor type, and donor-recipient sex combination (Gratwohl et al. 2009)

patient, disease stage, time interval from diagnosis to transplant, donor type, and donor-recipient sex combination were used to establish a score from 0 to 7 points. Its validity was tested in 56,505 patients, 33,113 (58%) male, 23,392 female, median age 33 years (range, 0.5–77 years), with an allogeneic HSCT for a hematological disorder between 1980 and 2005. The risk score was predictive in all disease categories, over all time periods, and was not altered by transplant techniques. Five well-defined pre-transplant patient and donor characteristics give a reasonable risk estimate of allogeneic HSCT. This risk score can provide a basis for the decision between transplant and non-transplant strategies.

26.5 Standardized Treatment, Technologies

26.5.1 Autologous Hematopoietic Stem Cell Transplantation (HSCT)

26.5.1.1 Conditioning Regimens

Stem cell transplantation requires always the preparation of the bone marrow compartment in particular but also the whole patient to receive the graft. The term "conditioning" has been coined for this central process of the stem cell transplantation which constitutes an integral part of the HSCT. However, there is an ongoing debate on how to bring the patient in the best condition possible to accept the graft.

The "creation of space" has been postulated as a major goal of conditioning. The original concept was that immature progenitor cells occupy circumscriptive bone marrow niches to gain the necessary support from feeder cells of the stroma for their proliferation and maturation. To this end the patient's stem cells have to be eradicated to provide the donor stem cells with access to these important niches so that engraftment might occur. The concept of reduced intensity conditioning (see below) with a fading in-fading out phenomena of donor stem cells versus host stem cells has at least to some extent relativated this concept. One might not necessarily have to eradicate the complete bone marrow to give a graft a realistic chance to engraft successfully. However, this depends on the underlying disease and the dynamic of the malignant clone.

In general the antitumor activity of the conditioning regimen is also needed to further reduce the tumor burden before transplantation. This is particularly true in autologous transplantation where no graft-versus-tumor effect is present.

For autologous HSCT standard conditioning regimens are in common practice according to the disease entity. For patients with multiple myeloma, 200 mg/m^2 melphalan has shown the best survival. For patients older than 60 years this dose might be reduced to 140 mg/m^2. Lymphoma patients (Hodgkin's disease (HD) and Non-Hodgkin-Lymphoma (NHL)) obtain most commonly a combination of carmustine (bis-chloronitrosourea; BCNU), etoposide, cytarabine and melphalan (Mills et al. 1995). Purging of transplants for lymphoma patients constitutes an interesting attempt, however it can be associated to hypoglobulinemia or secondary malignancies such as MDS or AML (Gyan et al. 2009). For patients with acute myeloid leukemia (AML), autologous HSCT has become rather rare, even more in Europe than in the US. The reasons are multiplex. In the last decade there has been no major progress in that field. Some centers report higher survival of patients receiving a purged transplant. But this has never been proven in a randomized trial. Most importantly, only half of the patients allocated to autologous HSCT for AML reach the transplantation because of relapse of the disease or a poor graft. For acute lymphoblastic leukemia the picture is even clearer. Several studies showed no difference for the comparison of chemotherapy versus autologous HSCT or even a significantly inferior outcome for auto-HSCT (Goldstone et al. 2008).

26.5.1.2 Mobilization

A standard protocol for the mobilization of autologous stem cells requires cyclophosphamide 1.5 g/m^2 on day 1 followed by 10 μg/kg BW/day G-CSF on days 2–12. Stem cells can be collected on days 10–12 when the WBC count reaches 8 G/L post nadir. In the case of poor mobilizers plerixafor (AMD3100) might be given in concert with G-CSF: on day 4 give additionally plerixafor at a dose of 160–240 μg/kg BW i.v. or i.m. 6–12 h before the intended harvest.

26.5.2 Allogeneic Hematopoietic Stem Cell Transplantation (HSCT)

26.5.2.1 Conditioning Regimens

The favorable results of allogeneic stem cell transplantation depend not only on chemotherapy and irradiation, but also on the allo-effect which is introduced by the graft from a family donor or unrelated donor. Therefore conditioning regimes for allogeneic HSCT must include immunosuppression to prevent a host-versus-graft reaction. Transplanted donor cells might be immediately attacked by immune cells of the host. Natural killer (NK) cells, T lymphocytes as well as dendritic cells (DCs) are involved in the complex interplay. There is a particular need for immunosuppression in the case of increasing human leukocyte antigen (HLA) disparities. The risk for graft rejection is also increased through pre-sensitization against minor histocompatibility antigens (miHAgs), e.g. through multiple blood product transfusions into the host preceding the allogeneic HSCT.

From the historical perspective, HSCT has been understood as a potential cure for patients irradiated through atomic bomb explosions or nuclear accidents. Therefore, total body irradiation (TBI; 12 Gray [Gy]) was tested first as conditioning method. TBI was efficient in eliminating the hematopoietic system, but TBI alone could not eradicate the leukemic clone. Only by adding cyclophosphamide (Cy) to TBI in patients at early stage disease, the first successful allogeneic HSCTs could be performed in the 1970ies. In further studies TBI was replaced by the "radiomimetic" busulfan (Bu), i.e. Bu/Cy. Other alkylating drugs like melphalan (Mel) or carmustine (BCNU), as well as "leukemia-specific" drugs like cytarabine (ARA-C), etoposide (ETO) and 6-thioguanine (6-TG) followed in a conditioning regimen termed BACT. As for TBI/Cy and Bu/Cy there are several differences in toxicities (more venous occlusive disease [VOD], more permanent alopecia with Bu/Cy), but both regimens are comparable in terms of long-term survival of the patients with the exception of ALL, where TBI/Cy is more effective.

With regard to the reduction of transplantation-related mortality (TRM) and the quality of life (QoL) the concept of "reduced intensity conditioning (RIC)" was born. In preclinical experiments the requirements for a stable engraftment were evaluated, and subsequently low and even lowest dose TBI (2–4 Gy) were used as well as conditioning regimens with fludarabine (Flu), Bu, Mel and Cy. RIC concepts became particularly interesting in the context of donor lymphocyte infusions (DLIs) which where inaugurated at the begin of the 1990s as a tool to bring patients with myeloid disease back into remission.

26.5.2.2 Graft-Versus-Host Disease (GvHD)

Graft-versus-host disease (GvHD) constitutes one of the most serious complications after allogeneic stem cell transplantation. The underlying pathomechanism is

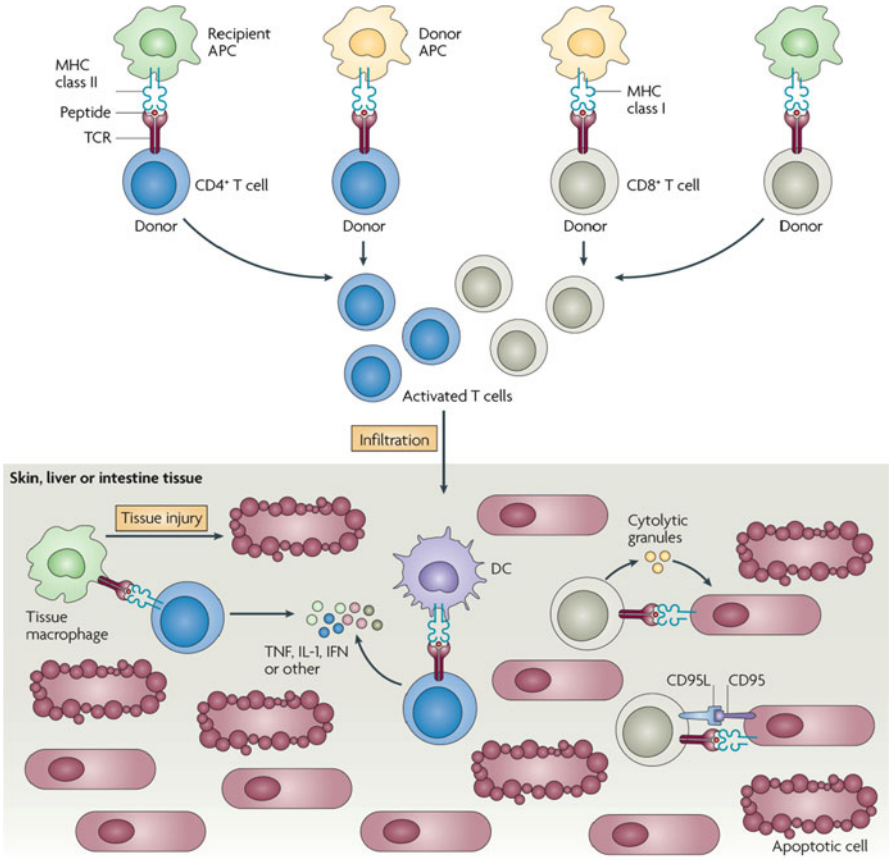


Fig. 26.10 GvHD – Mechanism of injury by tissue-infiltrating alloreactive T cells. Activated alloreactive CD8+ T cells require direct cognate interactions with MHC-class-I-expressing parenchymal targets (non-haematopoietic tissues such as skin, liver or intestine) to induce injury through the expression of CD95 ligand (CD95L) and the production of cytolytic granules. By contrast, infiltrating CD4+ T cells can mediate graft-versus-host disease (GVHD) without directly contacting MHC-class-II-expressing non-haematopoietic tissues. CD4+ T cells could be activated locally by tissue macrophages and dendritic cells (DCs) to release inflammatory mediators, such as tumour-necrosis factor (TNF), interleukin-1 (IL-1) and interferon (IFN), or alternatively may activate recipient antigen-bearing macrophages which induce tissue injury. *APC* antigen-presenting cell, *TCR* T-cell receptor {Shlomchik 2007}

the recognition of host-specific proteins by T cells of the donor which were transferred together with the donor stem cells or maturated thereof. Moreover dendritic cells of the host are involved in this complex interaction of the immune system depicted schematically in Fig. 26.10.

Clinical manifestations of GvHD can be detected on skin, liver, and GI tract. A macula-papular rash can develop on the upper part of chest and back as well as a

palmo-plantar exanthema. It might involve the entire integument and might lead to desquamation or even the development of bullae. Cholestatic hepatopathy with classical jaundice, increased serum levels of bilirubin as well as of cholestatic enzyme (whereas transaminases rather show no specific changes) are signs of GvDH of the liver. This needs to be differentiated from the veno-occlusive disease (VOD) which occurs rather early after allogeneic HSCT even before immunoreconstitution and subsequently the basis for a GvHD occurs. VOD is characterized by liver pain, ascites, impaired flow of intrahepatic veins and elevated serum levels of bilirubin and cytokeratin 17.

GvHD of the GI tract manifests as nausea, vomitus, loss of appetite, diarrhea and consequently weight.

The classical grading system inaugurated by Glucksberg et al. in 1974 stages four grades for each organ system according to the percent of integument with maculo-papular rash, the serum level of bilirubin and the quantity of diarrhea (Glucksberg et al. 1974).

Besides the acute type of GvHD described above which is defined as a GvHD starting within the first 100 days from the day of allogeneic HSCT, there exists also a chronic form of the disease. Chronic GvHD is characterized by the clinical feature of sclerodermia, Sjögren's syndrome, primary biliary cirrhosis, wasting syndrome, bronchiolitis obliterans (BO), as well as chronic cellular immunodeficiency.

Of note, the desirable Graft-versus-leukemia (GVL) effect seems to be interwoven with the noxious GvHD which can be explained by the fact that a general T cell activation can always results in an activation of an anti-leukemic T cell clone. Therefore, patients with a grade I to II GvHD show a better overall survival as the anti-leukemia/-lymphoma effect overweighs the immune aggression of the graft towards the recipient's organs.

26.5.2.3 Immunosuppressive Agents Against GvHD

In an attempt to alleviate the immune attack of the graft, an appropriate immunosuppressive prophylaxis as well as in the case of occurrence of GvHD an intensified immunosuppressive therapy is mandatory.

Standard drugs for GvHD prophylaxis include anti-thymoglobuline (ATG), campath (a monoclonal antibody against CD52, a pan-lymphocyte marker), methotrexate (MTX), cyclosporine A (CSA/CyA), mycophenolate mofetil (MMF) or its pro-drug MPA. Therapy of GVHD consist primarily of (methyl-) prednisolone, tacrolimus, sirolimus, everolimus, basiliximab/anti-interleukin-2, rituximab, blockers of tumor necrosis factor alpha (anti-TNFa), pentostatin. Extracorporeal photopheresis (ECP) can often cause tremendous improvement of GvHD without putting the patient at an increased risk for infectious disease. Mesenchymal stem cells (MCSs) are under current investigation for the treatment of GvHD and hold promise.

26.6 Clinical Experiences with Stem Cell Transplantation as a Paradigm of Regenerative Medicine

26.6.1 Genetic Diseases

26.6.1.1 Hemoglobinopathies (Thalassemia and Sickle Cell Disease [SCD])

Stem cell transplantation constitutes the only curative therapy for patients with thalassemia or sickle cell disease, although significant progress has been made in the field of supportive care such as oral therapy with iron chelators. Particularly in adulthood, severe affection of parenchymal organs by hemoglobinopathies is obvious and lifethreatening. According to the recommendations of the EBMT (Apperley et al. 2012) (http://www.ebmt.org/EBMT_Handbook.html), definite indications for HSCT constitute transfusion-dependent alpha- or beta-thalassemia major, transfusion-dependent HbE/beat-thalassemia when the patient is not older than 16 years and has a HLA-identical family donor. In special circumstances thalassemia in adult patients between 17 and 35 years might be considered.

Definite indications for HSCT in patients with sickle cell disease (SCD) depend on the existence of one or more of the following clinical complications: (1) SCD-related neurological deficit, stroke or subarachnoid hemorrhage. (2) Recurrent sickle chest syndrome or failure of response to therapy with hydroxyurea (HU) or contraindication of HU. (3) Recurrent and severe debilitating pain due to vaso-occlusice crises despite therapy with HU or contraindication of the drug (Amrolia et al. 2003a, b).

Conditioning regimens for hemoglobinopathies include Bu/Cy or Treosulfan/Cy in concert with campath/alemtuzumab or ATG. The transplantation-related mortality is very low with 2–5% and the risk of graft failure less than 5%. Three independent factors for the outcome of HSCT for thalassemia in children have been established by the Pesaro classification: hepatomegaly, portal fibrosis as diagnosed by liver biopsy and lack of compliance with iron chelation. By positive selection of CD34+ stem cells with a minimal contamination of T cells HSCT was also possible in the haploidentical setting from mother to child in the absence of a HLA-matched donor (Sodani et al. 2009).

For SCD there is no good score, however SCD patients who suffer from impaired quality of life (QoL) as stated above profit most from HSCT. The results of HCST for SCD are enchanting with an overall survival is very favorable with about 90%, a disease free survival about 85%, and both TRM and graft rejection <10% (Bhatia and Walters 2008).

26.6.1.2 Severe Combined Immunodeficiency (SCID)

SCID usually manifests as opportunistic infections in early childhood such as cytomegalovirus or fungal infections, or as absence of thymic shadow on chest X-ray films lymphocytopenia. Allogeneic HSCT constitutes the sole cure for these young

children and procures a survival rate of more than 90%. In the absence of an appropriate donor, MUD or haploidentical family donors are also acceptable. The usual conditioning regimen consists of Bu/Cy with the addition of ATG in the case of a MUD. As GvHD caused by the patients inability to reject the allogeneic cells, usually the graft is CD34-positive selected. However, the patients require a protective environment and anti-infective prophylaxis as well as intravenous substitution with immunoglobulins over at least 3 months. Due to the absence of B-cell engraftment in about 40% of the patients, these SCID patients require life-long immunoglobulin replacement therapy. In the meantime tremendous progress has been made and the results for haploidentical donors are as good as for HLA-genoidentical donor (Antoine et al. 2003).

Gene therapy for SCID-X1 patients has been undertaken with success (Hacein-Bey-Abina et al. 2003) as well as for SCID-ADA (Aiuti et al. 2009). Nevertheless many points regarding infectious complications and secondary neoplasia through vector integration are still poorly understood and need elucidation (Neven et al. 2009).

26.6.2 Aplastic Anemia

Aplastic anemia (AA) is a life-threatening disorder of the hematopoietic system characterized of bi- or pancytopenia. Severe AA (SAA) is defined by insufficient marrow production in at least two hematopoetic cell lines, and very severe AA (VSAA) if the neutrophil count is less than 0.2 G/l. Differential diagnosis of AA comprises paroxysmal nocturnal hemoglobinopathy (PNH), congenital dysplastic anemia (CDA), hypoplastic myelodysplastic syndrome (MDS). The ultimate goal of therapy for AA is freedom from transfusions. This can be achieve either by immunosuppressive therapy including CSA, ATG and prednisolone, or HSCT. The 5-year survival data are with 70–80% similar for both treatment options (Bacigalupo and Passweg 2009).

BMT is superior over peripheral blood HSCT in AA (Schrezenmeier et al. 2007). A survival benefit of BMT over mere immunosuppressive therapy (IS) has been established for patients younger than 40 years with VSAA and in patients in whom ISA failed.

The standard regimen for conditioning comprises Cy 200 mg/kg BW with or without ATG. With ATG less chronic GvHD and a better survival has been observed than with Cy alone (Storb et al. 1994). For patients older than 30 years, also non-myeloablative conditioning regimens with fludarabine have been successfully established (Piccin et al. 2009). There is rather limited information on the use of haploidentical donors and cord blood in patients with AA to make a definite statement at that time. The right immunosuppressive treatment after HSCT for AA is an ongoing debate. ATG, CSA and steroids are the standards. Some favor the addition of G-CSF to CSA and ATG. Recently, the supplier withdrew the equine ATG from the market and replaced it by rabbit ATG which is produced in the same manner, i.e. by stimulation with human thymocytes.

26.6.3 Myeloproliferative Neoplasias (MPN) of the Hematopoiesis

Chronic myeloid leukemia (CML) as one type of MPN is classically characterized by the translocation t(9;22) resulting in the Philadelphia chromosome has been considered as the paradigm of diseases which can be cured by HSCT. Through the advent of the tyrosinase kinase inhibitors (TKIs) such as imatinib mesylate (IM), dastinib and nilotinib, this feature has completely changed (Deininger 2008). IM has replaced interferon-alpha as first line therapy for CML. From the IRIS study the following results were reported: The cumulative best complete cytogenetic response (CCyR) rate was 82%; 63% of all patients randomized to receive imatinib and still on study treatment showed CCyR at last assessment. The estimated event-free survival at 6 years was 83%, and the estimated rate of freedom from progression to AP and BC was 93%. The estimated overall survival was 88% – or 95% when only CML-related deaths were considered. This 6-year update of IRIS underscores the efficacy and safety of imatinib as first-line therapy for patients with CML.

Therefore any decision on HSCT for CML has to take into account the following aspects: age of the patient, disease phase, duration of disease, nature of stem cell donor and the genders of recipient and donor, as well as the response of the patient to TKIs. As more and more TKIs like e.g. DCC-2036 targeting the difficult-to-treat mutation T315I (Chan et al. 2011) are available on the market one might suggest patients to use one TKI after the others as patients with HIV infection take different combinations of highly active anti-retroviral therapy (HAART) after the other, thus living with their disease for decades without dying from the disease. These considerations have been implemented in an EBMT risk factor score.

The role of allogeneic stem cell transplantation in chronic myeloid leukemia is being reevaluated. Whereas drug treatment has been shown to be superior in first line treatment, data on allo SCT as second line therapy after imatinib failure are scarce. Three year survival after transplantation of 56 patients in chronic phase was 91% (median follow-up 30 months). Transplantation related mortality was 8%. In a matched pair comparison of transplanted and non-transplanted patients, survival was not different. Three year survival after transplantation of 28 patients in advanced phase was 59%. Eighty eight percent of transplanted patients achieved complete molecular remissions. We conclude that allo SCT could become the preferred second line option after imatinib failure for suitable patients with a donor (Saussele et al. 2009).

Allogeneic HSCT is certainly not an option for CML patients in full manifest blast crisis; here newer TKIs are anyway more successful. After achieving a second chronic phase allogeneic HSCT is feasible.

Standard conditioning for HSCT in CML patients are Bu/Cy or TBI/Cy. An interesting aspect is that non-myeloablative HSCT followed by donor lymphocyte infusions (DLIs) for female patients with CML in fertile age open them a therapeutic option to bear a child after HSCT (Ringhoffer et al. 2007). This might require asservation of oocytes prior to HSCT. In contrast TKIs constitute an absolute contraindication to pregnancy because of their high teratogenic potential.

26.6.4 Acute Myeloid Leukemia (AML)

Acute myeloid leukemia (AML) constitutes a clonal disorder of the myeloid lineage which is associated with unlimited proliferation and loss of differentiation. AML is the most common adult leukemia with an incidence of about 3 per 100,000 inhabitants. Several characteristic chromosomal aberrations such as translocations t(8;21), t(15;17), t(9;11) and others can be detected in half of the patients. The majority of the remaining AML patients can be characterized by different mutations of genes like the *FMS-like tyrosine kinase type 3* gene (*FLT3*), the *mixed lineage leukemia* (*MLL*) gene, *the nucleophosmin 1* (*NPM1*) gene, the gene encoding *runt-related transcription factor 1* (*RUNX1*), the *tet oncogene family member 2* (*TET2*), and others (Dohner and Gaidzik 2011).

Several systems have been established to stratify AML patients according to these genetic abnormalities for their relapse risk. This risk stratification has led to treatment schedules with a different intensity of polychemotherapy to counterbalance the effects and side effects of cytostatic drugs used in these treatment algorithms (Dohner et al. 2010). To achieve a high rate of complete remissions (CR) in patients younger than 60 years old, the application of four chemotherapy cycles with cytarabine is considered to be essential (Dohner et al. 2010). Elderly patients with AML might be better treated by daunorubicin (Lowenberg et al. 2009). However, there is a big difference between CR which can be achieved in 60–80% of adults younger than 60 years on one hand, and the 25–30% 5-year survival rate of all AML patients. This clearly indicates that despite hematological CR, a sufficient number of leukemic cells can hibernate in their bone marrow niches to survive conventional chemotherapy. Here HSCT which combines chemotherapy, irradiation as well as the allo-effect can eradicate such hibernating leukemic (stem) cells (Koreth et al. 2009).

However, there is a transplantation-associated mortality (TAM) of 20–25% which is made up by opportunistic infections, by GvHD as well as by relapse of the disease. Standard conditioning consists of busulfan and cyclophosphamide or TBI, but phase III trials including fludarabine and treosulfane are also underway at our institution and others (Casper et al. 2010; Beelen et al. 2008).

In AML patients at relapse or with refractory disease, a conditioning regimen inaugurated by the group around Hans-Jochem Kolb has proven great efficacy with even up to 30% long-term survivors in this deleterious situation: after a block of fludarabine, amsacrine and cytarabine, the patient in aplasia receives a "non-myeloablative" conditioning with only 2×2 Gy TBI and standard dose of cyclophosphamide. The acronym FLAMSA has been coined for this particular conditioning approach (Schmid et al. 2006). Donor lymphocyte infusion might even improve the outcome of this approach if given at day +100 or later in the absence of GVHD (Schmid et al. 2007).

The data of the Acute Leukemia Working Party (ALWP) of the European Bone Marrow Transplantation (EBMT) Society demonstrate in a large cohort of 2,100 patients that autologous HSCT in patients with AML in 1st remission can result in

an outcome at 5 years with an overall survival of 51%, a relapse rate of 53% and a TAM of only 9% (Breems and Lowenberg 2007). The relapse rate is too high in this autologous setting. Moreover, it is not possible to collect enough stem cells in more than half of the AML patients and early relapse of the disease might even prevent autologous transplantation.

Therefore, the autologous approach has been given up in many centers and has been clearly surpassed by allogeneic HSCT. Patients at low cytogenetic risk and with a certain mutation status (NPM1pos. while FLT3-ITDneg.) might be sufficiently treated by chemotherapy. The majority of AML patients with intermediate cytogenetic risk allogeneic HSCT should be performed if a HLA-matched sibling is available for transplantation. Patient with high cytogenetic risk and a low or moderate co-morbidity score will profit from early transplantation in 1st CR. Patients at relapse of the disease will also profit from allogeneic HSCT. A special type of AML, the acute promyelocytic leukemia (APL; former AML FAB M3) characterized by the translocation t(15;17) is never recommended for HSCT.

26.6.5 Acute Lymphoblastic Leukemia (ALL)

Treatment results in adult acute lymphoblastic leukemia (ALL) have improved considerably in the past decade, with an increase of complete remission rates to 85–90% and overall survival rates to 40–50%. Superior chemotherapy and supportive care, the integration of hematopoietic stem cell transplantation (HSCT) into frontline therapy, and optimized risk stratification were important developments. Even more impressive is the success of targeted therapies in subgroups of ALL. In the formerly most unfavorable subgroup, Philadelphia chromosome (Ph)/BCR-ABL-positive ALL, survival now ranges from 40 to 50% after incorporating imatinib in combination chemotherapy. In mature B-ALL, survival rates increased above 80% with the combination of short intensive chemotherapy and rituximab. The prerequisite for comprehensive therapy is standardized and rapid diagnosis and classification as the basis for treatment stratification (Gokbuget and Hoelzer 2009).

Allogeneic hematopoietic stem cell transplantation as part of post-remission therapy improves survival for adult patients with high-risk acute lymphoblastic leukemia. A meta-analysis of seven studies including almost 1,300 patients demonstrated that patients in the donor groups had significantly better survival than patients in the no-donor groups (hazard ratio [HR], 1.29; 95% confidence interval [95% CI], 1.02–1.63 [P = .037]). In high-risk patients with Philadelphia-positive ALL, the superiority of the survival advantage was even greater (HR, 1.42; 95% CI, 1.06–1.90 [P = .019]). A meta-regression analysis revealed that compliance with allogeneic HSCT showed a significant and positive correlation with survival (coefficient, 0.022; P < .01). This suggests that allogeneic HSCT improves the outcome of adult patients with high-risk ALL. Allogeneic HSCT should be considered for such patients if a suitable donor is available (Yanada et al. 2006).

26.6.6 Chronic Lymphocytic Leukemia (CLL)

Chronic lymphocytic leukemia (CLL) constitutes the most common leukemia in western industry countries. In recent years both autologous and allogeneic HSCT have been exploited to treat CLL patients. Peter Dreger and others defined criteria for poor-risk CLL patient according to the EBMT CLL transplant consensus (Dreger et al. 2007): these are defined as non-response or early relapse (within 12 months) after purine analogue-containing therapy, relapse with 24 months after purine analogue combination therapy or treatment of similar efficacy (i.e. autologous HSCT) or p53 deletion/mutation (del17p13) requiring treatment. There is now only limited hope that autologous HSCT could cure CLL. Rather there is cumulating evidence that the graft-versus-leukemia (GVL) effect is crucial for the therapy of CLL by allogeneic HSCT.

Several leukemia-associated antigens (LAAs) have been identified in CLL patients (Giannopoulos et al. 2009). Allogeneic HSCT from matched related or unrelated donors can overcome the treatment resistance of poor-risk CLL as defined above. Even with reduced-intensity conditioning, allogeneic HSCT in CLL is associated with significant mortality and morbidity due to (chronic) GVHD, which has to be weighed against the risk of the disease when defining the indication for transplantation. Therefore, it can be regarded as a reasonable treatment option only for eligible patients who fulfill accepted criteria for poor-risk disease. If allogeneic HSCT is weighted, it should be performed before CLL has advanced to a status of complete refractoriness to assure an optimum chance for a successful outcome. Prospective trials are underway to prove whether allogeneic HSCT might indeed change the natural course of poor-risk CLL (Dreger 2009), particularly del17 patients and patients after the use of the anti-CD52 monoclonal antibody alemtuzumab (Brown 2011).

26.7 Conclusions and Future Perspectives on "*Blood*" Regenerative Therapies

Autologous and allogeneic hematopoietic stem cell transplantation is well established as a treatment modality to escalate antineoplastic therapy in some diseases and to replace deficient or neoplastic hematopoiesis in others. Alas, the results of autologous transplantation are hampered by a high incidence of relapses and the limiting barrier for the expansion of allogeneic transplantation is the increasing incidence of GvHD in older patients and with increasing HLA disparities.

In autologous transplantation the improvement of remission quality and the prolongation of remission duration by introduction of new anti-neoplastic agents into remission and maintenance is intensively studied. We will not extend on this perspective as it is not in the center of interest in regenerative medicine.

In allogeneic transplantation GvHD on one side and immune competence after transplant on the other side is correlated with the presence and absence of different immune cells in the graft. These cells become increasingly well defined and methods

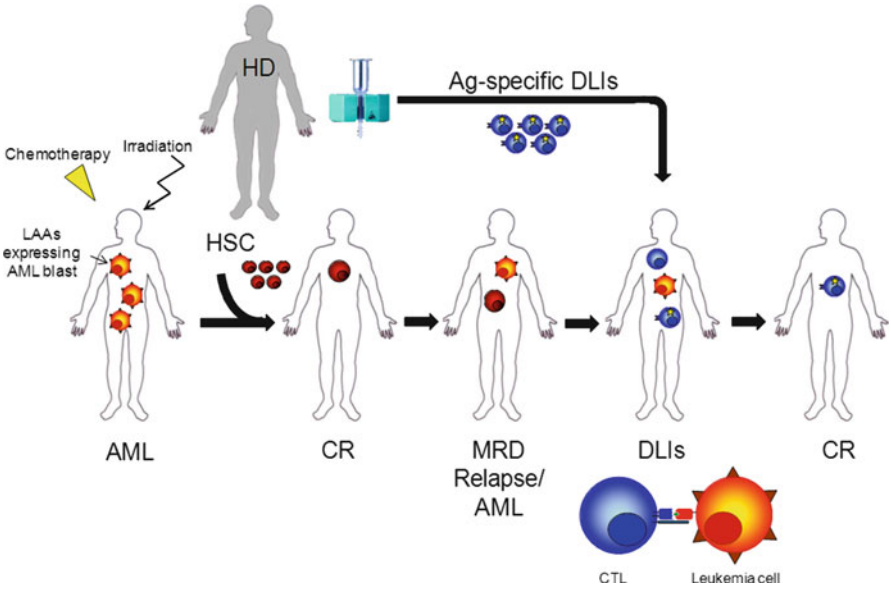


Fig. 26.11 GVL effect after HSCT and DLIs/Ag-specific CTLs. After chemotherapy and irradiation, HSC are infused for the re-population of the bone marrow. However, the graft exerts not only a GVL effect, but potentially also causes GvHD. In case of relapse, either DLIs or antigen-specific CTLs are infused with a good outcome against leukemia cells due to the GVL reaction. *LAA* leukemia associated antigen, *HD* healthy donor, *HSC* hematopoietic stem cell, *DLIs* donor lymphocyte infusions, *CR* complete remission, *MRD* minimal residual disease, *AML* acute myeloid leukemia (Taken from our recent publication Casalegno-Garduno et al. 2010)

are developed to isolate and manipulate them. Therefore much hope rests on engineered grafts for the improvement of allogeneic transplantation. The result of this development might be the elimination of virus infections after allogeneic transplantation and the enhancement of graft-versus-tumor effect.

26.7.1 Selective Donor Lymphocyte Infusions (sDLIs)

Multimers such as tetramers, pentamers, or streptamers, mimick the immunological synapses that naturally occur between T cells and antigen presenting cells (APC), through the T cell receptor (TCR) and major histocompatibility complex (MHC). Multimers are used to select either virus- or antigen-specific T cells (Casalegno-Garduno et al. 2010). However, only streptamers are currently available at GMP level.

Streptamer selection might facilitate the adoptive transfer of T cells, thus dissecting both GvHD from GVL effects. Streptamers can select either antigen- or virus-specific CTLs from seropositive donors (Fig. 26.11). This multimer is reversible and the CTLs remain functionally active.

We have own experience with the transfer of CMV-specific T cells into patients after allogeneic stem cell transplantation who suffered from recurrent CMV antigenemia. In two patients, the course of CMV antigenemia was definitively controlled after a single DLI with CMVpp65 specific CD8+ T cells (Schmitt et al. 2010).

26.7.2 Translation Towards Leukemia Antigen-Specific DLIs

In an ongoing study (Wang et al. 2010), we observed a significant difference in the frequency of WT1-specific CD8+ T cells in healthy donors and AML patients. It was possible to purify the WT1-specific T cells labeled with specific streptamers coupled with magnetic beads by MACS™ columns, and we achieved a purity of up to 90%. In our eyes this approach holds promise for clinical application in patients after allogeneic stem cell transplantation and detectable (minimal) residual disease to achieve a GVL effect without GvHD. This approach is currently extended to other LAAs.

26.7.3 Ways to Improve the Homeostatic Expansion of Antigen-Specific CD8+T Cells

With the paradigm of CMV-specific T cells we demonstrated that adoptively transferred antigen-specific cells can expand by homeostatic expansion in the periphery bypassing thymic priming (Surh and Sprent 2005). A similar homeostatic expansion of leukemia antigen-specific T cells is highly desirable. This might be facilitated by the depletion of CD4 + CD25hi regulatory T cells (Tregs), the shift in the balance of CD8 vs. CD4 + CD25hi Tregs. To this end several methods are under current investigation (Mielke et al. 2011) . Administration of T cells by cytostatic drugs like cyclophosphamide and fludarabin as well as total-body irradiation might cause at least to some extend lymphodepletion. Most importantly, vaccination against leukemia-antigens like WT1, PR1 or RHAMM (Schmitt et al. 2008) has been performed after chemotherapy and autologous stem cell transplantation. Now the challenge is to administer vaccines after allogeneic stem cell transplantation to booster the anti-leukemic GvL effect and to maintain this reaction for an extended time period. The format of post-transplantation vaccines (Rezvani 2011) might be a peptide (Rapoport et al. 2011) or a truncated protein. Dendritic cells transfected with RNA encoding the leukemia antigen (Van Tedeloo et al. 2010) constitute a further option as well as K562 cells transfected with the gene encoding granulocyte macrophage colony stimulating factor (GM-CSF), a vaccine designated GVAX.

Suggested reading for more information on all aspects of hematopoietic stem cell transplantation:

1. Apperley Jane, Carreras Enric, Gluckman Eliane, Gratwohl Alois, Masszi Tamás (eds) (2012) The EBMT handbook – haematopoietic stem cell transplantation, 6th edn. Forum Service Editore, Genoa. http://www.ebmt.org/EBMT_Handbook.html
2. Reinhold Munker, Hillard M. Lazarus, Kerry Atkinson (2013) The BMT data book, 3nd edn. Cambridge University Press, Cambridge. ISBN 978-1107617551 paperback
3. Hematology (2011) The American Society of Hematology education program book. http://asheducationbook.hematologylibrary.org
4. Kerry Atkinson, Richard Champlin, Jerome Ritz, Willem E. Fibbe, Per Ljungman, Malcom K. Brenner (2004) Clinical bone marrow and blood stem cell transplantation, 3rd edn. Cambridge University Press, Cambridge. ISBN 0-521-82912-7
5. E. Donnall Thomas, Karl G. Blume, Stephen J. Forman (1999) Hematopoietic cell transplantation, 2nd edn. Blackwell Science, Oxford. ISBN 978-0-632-04371-2

References

Aiuti A, Cattaneo F, Galimberti S, Benninghoff U, Cassani B, Callegaro L, Scaramuzza S, Andolfi G, Mirolo M, Brigida I, Tabucchi A, Carlucci F, Eibl M, Aker M, Slavin S, Al Mousa H, Al Ghonaium A, Ferster A, Duppenthaler A, Notarangelo L, Wintergerst U, Buckley RH, Bregni M, Marktel S, Valsecchi MG, Rossi P, Ciceri F, Miniero R, Bordignon C, Roncarolo MG (2009) Gene therapy for immunodeficiency due to adenosine deaminase deficiency. N Engl J Med 360:447–458

Amrolia PJ, Almeida A, Davies SC, Roberts IA (2003a) Therapeutic challenges in childhood sickle cell disease. Part 2: a problem-orientated approach. Br J Haematol 120:737–743

Amrolia PJ, Almeida A, Halsey C, Roberts IA, Davies SC (2003b) Therapeutic challenges in childhood sickle cell disease. Part 1: current and future treatment options. Br J Haematol 120:725–736

Antoine C, Muller S, Cant A, Cavazzana-Calvo M, Veys P, Vossen J, Fasth A, Heilmann C, Wulffraat N, Seger R, Blanche S, Friedrich W, Abinun M, Davies G, Bredius R, Schulz A, Landais P, Fischer A (2003) Long-term survival and transplantation of haemopoietic stem cells for immunodeficiencies: report of the European experience 1968-99. Lancet 361:553–560

Apperley J, Carreras E, Gluckman E, Gratwohl A, Masszi T (2012) The EBMT-ESH handbook – haematopoietic stem cell transplantation, 6th edn. Forum Service Editore, Genoa

Arachchillage DR, Dalley CD, Reilly JT, Wilson G, Collins N, Snowden JA (2010) Long-term dual donor derived haematopoietic reconstitution following double unrelated cord blood transplantation – single unit dominance is not always the case. Br J Haematol 149(2):298–299

Bacigalupo A, Passweg J (2009) Diagnosis and treatment of acquired aplastic anemia. Hematol Oncol Clin North Am 23:159–170

Barker JN, Scaradavou A, Stevens CE (2009) Combined effect of total nucleated cell dose and HLA-match on transplant outcome in 1061 cord blood recipients with hematological malignancies. Blood 115(9):1843–1849

Barnes DW, Loutin JF (1953) Protective effects of implants of splenic tissue. Proc R Soc Med 46:251–252

Beelen DW, Gromke T, Trenschel R, Hilgendorf I, Wolff D, Elmaagacli A, Freund M, Casper J (2008) Treosulfan compared to total body irradiation-based preparative regimens before allogeneic stem cell transplantation for acute myeloid leukemia: a retrospective long-term study. ASH Ann Meet Abs 112(11):2149

Bhatia M, Walters MC (2008) Hematopoietic cell transplantation for thalassemia and sickle cell disease: past, present and future. Bone Marrow Transplant 41:109–117

Bhattacharya D, Czechowicz A, Ooi AG, Rossi DJ, Bryder D, Weissman IL (2009) Niche recycling through division-independent egress of hematopoietic stem cells. J Exp Med 206:2837–2850

Bortin MM (1970) A compendium of reported human bone marrow transplants. Transplantation 9:571–587

Bradley TR, Metcalf D (1966) The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci 44:287–300

Breems DA, Lowenberg B (2007) Acute myeloid leukemia and the position of autologous stem cell transplantation. Semin Hematol 44:259–266

Brown JR (2011) The treatment of relapsed refractory chronic lymphocytic leukemia. Hematol Am Soc Hematol Educ Prog 2011:110–118

Casalegno-Garduno R, Schmitt A, Yao J, Wang X, Xu X, Freund M, Schmitt M (2010) Multimer technologies for detection and adoptive transfer of antigen-specific T cells. Cancer Immunol Immunother 59:195–202

Casper J, Wolff D, Knauf W, Blau IW, Ruutu T, Volin L, Wandt H, Schäfer-Eckart K, Holowiecki J, Giebel S, Aschan J, Zander AR, Kröger N, Hilgendorf I, Baumgart J, Mylius HA, Pichlmeier U, Freund M (2010) Allogeneic hematopoietic stem-cell transplantation in patients with hematologic malignancies after dose-escalated treosulfan/fludarabine conditioning. J Clin Oncol 28:3344–3351

Chan WW, Wise SC, Kaufman MD, Ahn YM, Ensinger CL, Haack T, Hood MM, Jones J, Lord JW, Lu WP, Miller D, Patt WC, Smith BD, Petillo PA, Rutkoski TJ, Telikepalli H, Vogeti L, Yao T, Chun L, Clark R, Evangelista P, Gavrilescu LC, Lazarides K, Zaleskas VM, Stewart LJ, Van Etten RA, Flynn DL (2011) Conformational control inhibition of the BCR-ABL1 tyrosine kinase, including the gatekeeper T315I mutant, by the switch-control inhibitor DCC-2036. Cancer Cell 19:556–568

Colvin GA, Lambert JF, Abedi M, Hsieh CC, Carlson JE, Stewart FM, Quesenberry PJ (2004) Murine marrow cellularity and the concept of stem cell competition: geographic and quantitative determinants in stem cell biology. Leukemia 18:575–583

Czechowicz A, Kraft D, Weissman IL, Bhattacharya D (2007) Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches. Science 318:1296–1299

Deininger MW (2008) Milestones and monitoring in patients with CML treated with imatinib. Hematol Am Soc Hematol Educ Prog 1:419–426

den Haan JM, Meadows LM, Wang W, Pool J, Blokland E, Bishop TL, Reinhardus C, Shabanowitz J, Offringa R, Hunt DF, Engelhard VH, Goulmy E (1998) The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. Science 279:1054–1057

Dohner H, Gaidzik VI (2011) Impact of genetic features on treatment decisions in AML. Hematol Am Soc Hematol Educ Prog 2011:36–42

Dohner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK, Dombret H, Fenaux P, Grimwade D, Larson RA, Lo-Coco F, Naoe T, Niederwieser D, Ossenkoppele GJ, Sanz MA, Sierra J, Tallman MS, Lowenberg B, Bloomfield CD (2010) Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood 115:453–474

Dreger P (2009) Allotransplantation for chronic lymphocytic leukemia. Hematol Am Soc Hematol Educ Prog 2009:602–609

Dreger P, Corradini P, Kimby E, Michallet M, Milligan D, Schetelig J, Wiktor-Jedrzejczak W, Niederwieser D, Hallek M, Montserrat E (2007) Indications for allogeneic stem cell transplantation in chronic lymphocytic leukemia: the EBMT transplant consensus. Leukemia 21:12–17

Ema H, Takano H, Sudo K, Nakauchi H (2000) In vitro self-renewal division of hematopoietic stem cells. J Exp Med 192:1281–1288

Ford CE, Hammerton JL, Barnes DW, Loutin JF (1956) Cytological identification of radiation-chimaeras. Nature 177:452–454

Giannopoulos K, Mertens D, Buhler A, Barth TF, Idler I, Moller P, Krober A, Greiner J, Chocholska S, Dmoszynska A, Rolinski J, Dohner H, Stilgenbauer S, Schmitt M (2009) The candidate immunotherapeutical target, the receptor for hyaluronic acid-mediated motility, is associated with proliferation and shows prognostic value in B-cell chronic lymphocytic leukemia. Leukemia 23:519–527

Ginsburg H, Sachs L (1963) Formation of pure suspension of mast cells in tissue culture by differentiation of lymphoid cells from the mouse thymus. J Natl Cancer Inst 31:1–40

Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, Lerner KG, Thomas ED (1974) Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation 18:295–304

Gokbuget N, Hoelzer D (2009) Treatment of adult acute lymphoblastic leukemia. Semin Hematol 46:64–75

Goldstone AH, Richards SM, Lazarus HM, Tallman MS, Buck G, Fielding AK, Burnett AK, Chopra R, Wiernik PH, Foroni L, Paietta E, Litzow MR, Marks DI, Durrant J, McMillan A, Franklin IM, Luger S, Ciobanu N, Rowe JM (2008) In adults with standard-risk acute lymphoblastic leukemia, the greatest benefit is achieved from a matched sibling allogeneic transplantation in first complete remission, and an autologous transplantation is less effective than conventional consolidation/maintenance chemotherapy in all patients: final results of the International ALL Trial (MRC UKALL XII/ECOG E2993). Blood 111:1827–1833

Goulmy E, Schipper R, Pool J, Blokland E, Falkenburg JH, Vossen J, Gratwohl A, Vogelsang GB, van Houwelingen HC, Van Rood JJ (1996) Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. N Engl J Med 334:281–285

Gratwohl A, Stern M, Brand R, Apperley J, Baldomero H, De Witte T, Dini G, Rocha V, Passweg J, Sureda A, Tichelli A, Niederwieser D (2009) Risk score for outcome after allogeneic hematopoietic stem cell transplantation: a retrospective analysis. Cancer 115:4715–4726

Gyan E, Foussard C, Bertrand P, Michenet P, Le Gouill S, Berthou C, Maisonneuve H, Delwail V, Gressin R, Quittet P, Vilque JP, Desablens B, Jaubert J, Ramee JF, Arakelyan N, Thyss A, Molucon-Chabrot C, Delepine R, Milpied N, Colombat P, Deconinck E (2009) High-dose therapy followed by autologous purged stem cell transplantation and doxorubicin-based chemotherapy in patients with advanced follicular lymphoma: a randomized multicenter study by the GOELAMS with final results after a median follow-up of 9 years. Blood 113:995–1001

Hacein-Bey-Abina S, von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint BG, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, MacIntyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302:415–419

Harrison DE, Astle CM (1982) Loss of stem cell repopulating ability upon transplantation. Effects of donor age, cell number, and transplantation procedure. J Exp Med 156:1767–1779

Klein J, Sato A (2000a) The HLA system. First of two parts. N Engl J Med 343:702–709

Klein J, Sato A (2000b) The HLA system. Second of two parts. N Engl J Med 343:782–786

Kodera Y (2008) The Japan marrow donor program, the Japan cord blood bank network and the Asia blood and marrow transplant registry. Bone Marrow Transplant 42(Suppl 1):S6

Koreth J, Schlenk R, Kopecky KJ, Honda S, Sierra J, Djulbegovic BJ, Wadleigh M, DeAngelo DJ, Stone RM, Sakamaki H, Appelbaum FR, Dohner H, Antin JH, Soiffer RJ, Cutler C (2009) Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. JAMA 301:2349–2361

Lowenberg B, Ossenkoppele GJ, van Putten W, Schouten HC, Graux C, Ferrant A, Sonneveld P, Maertens J, Jongen-Lavrencic M, Lilienfeld-Toal M, Biemond BJ, Vellenga E, van Marwijk

KM, Verdonck LF, Beck J, Dohner H, Gratwohl A, Pabst T, Verhoef G (2009) High-dose daunorubicin in older patients with acute myeloid leukemia. N Engl J Med 361:1235–1248
Mielke S, McIver ZA, Shenoy A, Fellowes V, Khuu H, Stroncek DF, Leitman SF, Childs R, Battiwalla M, Koklanaris E, Haggerty J, Savani BN, Rezvani K, Barrett AJ (2011) Selectively T cell-depleted allografts from HLA-matched sibling donors followed by Low-dose posttransplantation immunosuppression to improve transplantation outcome in patients with hematologic malignancies. Biol Blood Marrow Transplant 17:1855–1861
Mills W, Chopra R, McMillan A, Pearce R, Linch DC, Goldstone AH (1995) BEAM chemotherapy and autologous bone marrow transplantation for patients with relapsed or refractory non-Hodgkin's lymphoma. J Clin Oncol 13:588–595
Moretta A, Pende D, Locatelli F, Moretta L (2009) Activating and inhibitory killer immunoglobulin-like receptors (KIR) in haploidentical haemopoietic stem cell transplantation to cure high-risk leukaemias. Clin Exp Immunol 157:325–331
Morrison SJ, Prowse KR, Ho P, Weissman IL (1996) Telomerase activity in hematopoietic cells is associated with self-renewal potential. Immunity 5:207–216
Mutis T, Gillespie G, Schrama E, Falkenburg JH, Moss P, Goulmy E (1999) Tetrameric HLA class I-minor histocompatibility antigen peptide complexes demonstrate minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease. Nat Med 5:839–842
Neven B, Leroy S, Decaluwe H, Le Deist F, Picard C, Moshous D, Mahlaoui N, Debre M, Casanova JL, Dal Cortivo L, Madec Y, Hacein-Bey-Abina S, de Saint BG, de Villartay JP, Blanche S, Cavazzana-Calvo M, Fischer A (2009) Long-term outcome after hematopoietic stem cell transplantation of a single-center cohort of 90 patients with severe combined immunodeficiency. Blood 113:4114–4124
Nowell PC, Cole LJ, Habermeyer JG, Roan PL (1956) Growth and continued function of rat marrow cells in x-radiated mice. Cancer Res 16:258–261
Papayannopoulou T, Scadden DT (2008) Stem-cell ecology and stem cells in motion. Blood 111:3923–3930
Piccin A, McCann S, Socie G, Oneto R, Bacigalupo A, Locasciulli A, Marsh J, Schrezenmeier H, Tichelli A, Hand E, Lawler M, Passweg J (2009) Survival of patients with documented autologous recovery after SCT for severe aplastic anemia: a study by the WPSAA of the EBMT. Bone Marrow Transplant 45(6):1008–1013
Rammensee HG, Falk K, Rotzschke O (1993) Peptides naturally presented by MHC class I molecules. Annu Rev Immunol 11:213–244
Rapoport AP, Aqui NA, Stadtmauer EA, Vogl DT, Fang HB, Cai L, Janofsky S, Chew A, Storek J, Akpek G, Badros A, Yanovich S, Tan MT, Veloso E, Pasetti MF, Cross A, Philip S, Murphy H, Bhagat R, Zheng Z, Milliron T, Cotte J, Cannon A, Levine BL, Vonderheide RH, June CH (2011) Combination immunotherapy using adoptive T-cell transfer and tumor antigen vaccination on the basis of hTERT and survivin after ASCT for myeloma. Blood 117:788–797
Rezvani K (2011) Posttransplantation vaccination: concepts today and on the horizon. Hematol Am Soc Hematol Educ Prog 2011:299–304
Ringhoffer M, Harsdorf S, Schmitt M, Wiesneth M, Zenz T, Stilgenbauer S, Greiner J, Dohner K, Marx M, Dohner H, Bunjes D (2007) Reduced-intensity conditioning followed by T-cell depleted allogeneic stem cell transplantation for patients with chronic myeloid leukaemia and minimal residual disease at the time of transplant: high risk of molecular relapse. Br J Haematol 136:127–130
Saussele S, Lauseker M, Gratwohl A, Beelen DW, Bunjes D, Schwerdtfeger R, Kolb HJ, Ho AD, Falge C, Holler E, Schlimok G, Zander AR, Arnold R, Kanz L, Dengler R, Haferlach C, Schlegelberger B, Pfirrmann M, Muller MC, Schnittger S, Leitner A, Pletsch N, Hochhaus A, Hasford J, Hehlmann R (2009) Allogeneic hematopoietic stem cell transplantation (alloSCT) for chronic myeloid leukemia in the imatinib era; evaluation of its impact within a subgroup of the randomized German CML Study IV. Blood 115(10):1880–1885
Schmid C, Schleuning M, Schwerdtfeger R, Hertenstein B, Mischak-Weisinger E, Bunjes D, von Harsdorf S, Scheid C, Holtick U, Greinix H, Keil F, Schneider B, Sandherr M, Bug G, Tischer J, Ledderose G, Hallek M, Hiddemann W, Kolb H-J (2006) Longterm survival in refractory

acute myeloid leukemia after sequential treatment with chemotherapy and reduced-intensity conditioning for allogeneic stem cell transplantation. Blood 108:1092–1099

Schmid C, Labopin M, Nagler A, Bornhauser M, Finke J, Fassas A, Volin L, Gurman G, Maertens J, Bordigoni P, Holler E, Ehninger G, Polge E, Gorin NC, Kolb HJ, Rocha V (2007) Donor lymphocyte infusion in the treatment of first hematological relapse after allogeneic stem-cell transplantation in adults with acute myeloid leukemia: a retrospective risk factors analysis and comparison with other strategies by the EBMT Acute Leukemia Working Party. J Clin Oncol 25:4938–4945

Schmitt M, Schmitt A, Rojewski M, Chen J, Götz M, Heyduk M, Giannopoulos K, Ritter G, Gnjatic S, Guillaume P, Ringhoffer M, Schlenk R, Liebisch P, Bunjes D, Shiku H, Döhner H, Greiner J (2008) RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma elicits immunological and clinical responses. Blood 111:1357–1365

Schmitt A, Tonn T, Busch D, Grigoleit G, Einsele H, Odenthal M, Germeroth L, Ringhoffer M, Ringhoffer S, Wiesneth M, Detlef M, Mertens T, Rojewski M, Marx M, Harsdorf S, Döhner H, Seifried E, Bunjes D, Schmitt M (2010) Adoptive transfer and selective reconstitution of streptamer-selected CMV-specific CD8+ T cells leads to virus clearance in patients after allo-PBSCT. Transfusion 51:591–599

Schrezenmeier H, Passweg JR, Marsh JC, Bacigalupo A, Bredeson CN, Bullorsky E, Camitta BM, Champlin RE, Gale RP, Fuhrer M, Klein JP, Locasciulli A, Oneto R, Schattenberg AV, Socie G, Eapen M (2007) Worse outcome and more chronic GVHD with peripheral blood progenitor cells than bone marrow in HLA-matched sibling donor transplants for young patients with severe acquired aplastic anemia. Blood 110:1397–1400

Shlomchik WD (2007) Graft-versus-host disease. Nat Rev Immunol 7:340–352

Sodani P, Isgro A, Gaziev J, Polchi P, Paciaroni K, Marziali M, Simone MD, Roveda A, Montuoro A, Alfieri C, De Angelis G, Gallucci C, Erer B, Isacchi G, Zinno F, Adorno G, Lanti A, Faulkner L, Testi M, Andreani M, Lucarelli G (2009) Purified T-depleted, CD34+ peripheral blood and bone marrow cell transplantation from haploidentical mother to child with thalassemia. Blood 115(6):1296–1302

Storb R, Etzioni R, Anasetti C, Appelbaum FR, Buckner CD, Bensinger W, Bryant E, Clift R, Deeg HJ, Doney K (1994) Cyclophosphamide combined with antithymocyte globulin in preparation for allogeneic marrow transplants in patients with aplastic anemia. Blood 84:941–949

Surh CD, Sprent J (2005) Regulation of mature T cell homeostasis. Semin Immunol 17:183–190

Thomas ED, Lochte HL Jr, Lu WC, Ferrebee JW (1957) Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. N Engl J Med 257:491–496

Thomas E, Storb R, Clift RA, Fefer A, Johnson FL, Neiman PE, Lerner KG, Glucksberg H, Buckner CD (1975) Bone-marrow transplantation. N Engl J Med 292(832–43):895–902

Trowsdale J, Campbell RD (1988) Physical map of the human HLA region. Immunol Today 9:134–137

Van Tedeloo VF, Van de Velde V, Van Driesche A et al (2010) Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. Proc Natl Acad Sci USA 107:13824–13829

Vos O, Davids JA, Weyzen WW, van Bekkum DW (1956) Evidence for the cellular hypothesis in radiation protection by bone marrow cells. Acta Physiol Pharmacol Neerl 4:482–486

Wang X, Schmitt A, Chen B, Xu X, Mani J, Linnebacher M, Freund M, Schmitt M (2010) Streptamer-based selection of WT1-specific CD8+ T cells for specific donor lymphocyte infusions. Exp Hematol 38:1066–1073

Yanada M, Matsuo K, Suzuki T, Naoe T (2006) Allogeneic hematopoietic stem cell transplantation as part of postremission therapy improves survival for adult patients with high-risk acute lymphoblastic leukemia: a metaanalysis. Cancer 106:2657–2663

Chapter 27
Regenerative Medicine in the Central Nervous System: Stem Cell-Based Cell- and Gene-Therapy

Seung U. Kim

Abstract Human neurological diseases such as Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), multiple sclerosis (MS), stroke and spinal cord injury are caused by a loss of neurons and glial cells in the brain or spinal cord. Cell replacement therapy and gene transfer to the diseased or injured brain have provided the basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of human neurological diseases. However, the paucity of suitable cell types for cell replacement therapy in patients suffering from neurological disorders has hampered the development of this promising therapeutic approach. In recent years, neurons and glial cells have successfully been generated from stem cells such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) and neural stem cells (NSCs), and extensive efforts by investigators to develop stem cell-based brain transplantation therapies have been carried out. I review here notable experimental and pre-clinical studies previously published involving stem cell-based cell- and gene-therapies for PD, HD, ALS, AD, MS and stroke, and discuss for future prospect for the stem cell therapy of neurological disorders in clinical setting. There are still many obstacles to be overcome before clinical application of cell- and gene-therapy in neurological disease patients is adopted: (i) it is still uncertain how to generate specific cell types of neurons or glia suitable for cellular grafts in great quantity, (ii) it is required to abate safety concern related to tumor formation following NSC transplantation, and (iii) it needs to be better understood by what mechanism transplantation of NSCs leads to an enhanced functional recovery. Steady and stepwise progress in stem cell research in

S.U. Kim, M.D., Ph.D. (✉)
Division of Neurology, Department of Medicine, UBC Hospital, University of British Columbia, Vancouver, BC V6T 2B5, Canada

Medical Research Institute, Chung-Ang University College of Medicine, Seoul 156-756, South Korea
e-mail: sukim2005@gmail.com

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_27,

both basic and pre-clinical settings should support the hope for development of stem cell-based therapies for neurodegenerative diseases. This review focuses on the utility of stem cells particularly NSCs as substrates for structural and functional repair of the diseased or injured brain.

27.1 Introduction

In adult mammalian central nervous system (CNS), injured neurons exhibit low spontaneous capacity for regeneration (Ramon y Cajal 1928), although earlier studies have demonstrated regeneration in the injured adult CNS using fetal brain cells as transplants (Thompson 1890; Ransom 1909; Le Gros Clark 1940; cited by Kordower and Tuszinski 1999). It is important to adopt fetal CNS tissues as doners to promote repair in adult CNS since adult CNS cells do not survive in the grafted site. However, little progress has been seen for a long time in investigation related to the CNS regeneration until late 1970s. In 1979 two Swedish research groups reported independently that embryonic rat mesencephalic cells transplanted in the brain of parkinsonian rats survived and induced functional recovery (Björklund and Stenevi 1979; Perlow et al. 1979). This is the first time that fetal CNS cell transplants replace lost cells and restore functional deficits in animal models of neurological diseases. Since then, fetal CNS-derived cells were grafted into the brain and spinal cord of animal models of neurological disorders including Parkinson's disease (PD), Huntington's disease (HD), stroke and spinal cord injury. Starting late 1980s, transplantation of human fetal ventral mesencephalic tissues into the striatum of PD patients has been adopted as a successful therapy for patients with advanced disease (Lindvall et al. 1990; Olanow et al. 1996; Kordower et al. 1997a; Dunnett and Bjorklund 1999). However, this fetal brain tissue transplantation has grave problems associated with ethical and religious questions and limited supply of fetal tissues. To circumvent these difficulties, utilization of neurons with dopamine (DA) phenotype generated from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) or neural stem cells (NSCs) could serve as a practical and effective alternative for the fetal brain tissues for brain transplantation.

Cell replacement therapy and gene transfer to the diseased or injured brain have provided the basis for the development of potentially powerful new therapeutic strategies for human neurological diseases. However, the paucity of suitable cell types for cell therapy in patients suffering from neurological disorders has hampered the development of this promising therapeutic approach. In recent years, neurons and glial cells have successfully been generated from stem cells such as ESCs, iPSCs, MSCs and NSCs, and extensive efforts by investigators to develop stem cell-based brain transplantation therapies have been carried out.

Stem cells are defined as cells that have the ability to renew themselves continuously and possess pluripotent ability to differentiate into many cell types. Two types of mammalian pluripotent stem cells, ESCs derived from the inner cell mass

of blastocysts and embryonic germ cells (EGCs) obtained from post-implantation embryos, have been identified and these stem cells give rise to various organs and tissues (Thompson et al. 1998; Shamblott et al. 1998). Recently there has been an exciting development in generation of a new class of pluripotent stem cells, induced pluripotent stem cells (iPSCs), from adult somatic cells such as skin fibroblasts by introduction of embryogenesis-related genes (Takahashi et al. 2007; Yu et al. 2007; Park et al. 2008). In addition to ESCs and iPS cells, tissue specific stem cells could be isolated from various tissues of more advanced developmental stages such as hematopoietic stem cells (HSCs), bone marrow MSCs, adipose tissue-derived stem cells (ADSCs), amniotic fluid stem cells and NSCs. Among these, existence of multipotent NSCs has been known in developing or adult rodent brain with properties of indefinite growth and multipotent potential to differentiate into three major cell types of CNS, neurons, astrocytes and oligodendrocytes (McKay 1997; Flax et al. 1998; Gage 2000; Temple 2001; Gottlieb 2002; Kim 2004; Kim and deVellis 2009).

In human, existence of NSCs with multipotent differentiation capability has also been reported in embryonic and adult human brain (Flax et al. 1998; Kim 2004; Brustle and McKay 1996; Sah et al. 1997). In a group of cancer patients who had infusion of chemical bromodeoxyuridine (BrdU) for diagnostic purposes and later died, evidence that new neurons are continuously being generated in adult human CNS has been demonstrated (Eriksson et al. 1998). Why then there is only limited capacity to repair in adult CNS suffering from injury or diseases? It appears that endogenous brain environment that is responsible for induction of NSC proliferation and consequent NSC differentiation into neurons is not adequate in most of diseased or injured brain.

Recently continuously dividing immortalized cell lines of NSCs have been generated by introduction of oncogenes and these immortalized NSC lines have advantageous characteristics for basic studies on neural development and cell replacement therapy or gene therapy studies: (i) Stable immortalized NSC cells are homogeneous since they were generated from a single cell, i.e. a single clone; (ii) immortal NSC cells can be expanded readily in large numbers in short time; (iii) stable expression of therapeutic genes can be achieved readily (Flax et al. 1998; Kim 2004; Renfranz et al. 1991; Snyder et al. 1992; Lee et al. 2007a). Immortalized NSCs have emerged as highly effective source for genetic manipulation and gene transfer into the CNS *ex vivo*; immortalized NSCs were genetically manipulated *in vitro*, survive, integrate into host tissues and differentiate into both neurons and glial cells after transplantation to the intact or damaged brain. We have previously generated immortalized cell lines of human NSCs by infecting fetal human brain cells grown in primary culture with a retroviral vector carrying v-myc oncogene and selecting continuously dividing NSC clones. Both *in vivo* and *in vitro* these cells were able to differentiate into neurons and glial cells and populate the developing or degenerating CNS (Flax et al. 1998; Kim 2004; Lee et al. 2007a; Kim and de Vellis 2009).

Stem cell-based cell and gene therapy could serve as potentially powerful new therapeutic strategies for a broad spectrum of human neurological diseases including PD, HD, AD, ALS, MS, stroke, spinal cord injury and brain tumors (Brustle and

McKay 1996; Flax et al. 1998; Kim 2004; Lindvall et al. 2004; Goldman 2005; Kim and deVellis 2009). There are still many obstacles to be overcome before clinical application of cell therapy in neurological disease patients is adopted: (i) it is still uncertain how to generate specific cell types of neurons or glia suitable for cellular grafts in great quantity, (ii) it is required to abate safety concern related to tumor formation following NSC transplantation, and (iii) it needs to be better understood by what mechanism transplantation of NSCs leads to an enhanced functional recovery. Steady and stepwise progress in stem cell research in both basic and pre-clinical settings should support the hope for development of stem cell-based therapies for neurodegenerative diseases. This review focuses on the utility of stem cells particularly NSCs and MSCs as substrates for structural and functional repair of the diseased or injured CNS.

27.2 Parkinson Disease

Parkinson's disease (PD) is characterized by an extensive loss of dopamine neurons (DA) in the substantia nigra pars compacta and their terminals in the striatum (Kish et al. 1988; Agid 1991), and affects more than 500,000 people in the US. While the etiology of idiopathic PD is not known, several predisposing factors for the dopaminergic depletion associated with the disease have been suggested, including programmed cell death, viral infection, and environmental toxins. As an effective treatment for PD, patients have been given L-dihydroxyphenyl alanine (L-DOPA), a precursor of dopamine, but long-term administration of L-DOPA consequently produces grave side effects (Lang and Lozano 1998a, b). More recently surgical procedure of deep brain stimulation has been adopted as a successful treatment for PD patients (Lyons 2011).

Since late 1980s, transplantation of human fetal ventral mesencephalic tissues into the striatum of PD patients has been adopted as a successful therapy for patients with advanced disease (Lindvall et al. 1990; OLanow et al. 1996; Kordower et al. 1997a; Dunnett and Bjorklund 1999). However, this fetal tissue transplantation has grave problems associated with ethical and religious questions and logistics of acquiring fetal tissues. In addition, recent reports have indicated that the survival of transplanted fetal mesencephalic cells in the patients' brain was very low and it was difficult to obtain enough fetal tissues needed for transplantation (Hagell et al. 1999). To circumvent these difficulties, utilization of neurons with dopamine (DA) phenotype generated from ESCs, iPSCs, MSCs or NSCs could serve as a practical and effective alternative for the fetal brain tissues for transplantation (Lee et al. 2000). DA neurons were generated from mouse ESCs after treatment with fibroblast growth factor 8 (FGF8) and sonic hedgehog (Hagell and Brundin 2002), over-expression of Nurr1 (Wagner et al. 1999; Chung et al. 2002; Kim et al. 2003) or Bcl-XL (Shim et al. 2004), or co-culture with a mouse bone marrow stromal cell line (Kawasaki et al. 2000). Neurons with DA phenotype have been generated from monkey ESCs by co-culturing with mouse bone marrow stromal cells and behavioral improvement

was seen in MPTP-lesioned monkeys following intra-striatal transplantation of these cells (Takagi et al. 2005). DA neurons were also generated from neural progenitor cells derived from fetal brain and induced functional recovery following brain transplantation in parkinsoinian monkeys (Takagi et al. 2005).

Transplantation of NSCs in the brain attenuates anatomic or functional deficits associated with injury or disease in the CNS via cell replacement, the release of specific neurotransmitters, and the production of neurotrophic factors that protect injured neurons and promote neuronal growth. Recently we have generated continuously dividing immortalized cell lines of human NSC from fetal human brain cell culture via a retroviral vector encoding v-*myc* (Kim 2004; Lee et al. 2007a; Kim et al. 2008c) and one of the immortalized NSC lines, HB1.F3, induced functional improvement in rat model of PD following transplantation into the striatum (Yasuhara et al. 2006).

Earlier studies have used gene transfer technology to develop treatment for PD by transferring tyrosine hydroxylase (TH) gene, a rate-limiting step enzyme in catecholamine biosynthesis process, into certain cell types and then implant these cells into the brain of PD animal models (Wolff et al. 1989; Fisher et al. 1991; Jiao et al. 1993; Anton et al. 1994; During et al. 1994). However, gene transfer of TH using genetically modified cells produced only partial restoration of behavioral and biochemical deficits in PD animal models, since the cells utilized did not carry sufficient amount of tetrahydrobiopterin (BH_4), a cofactor to support TH activity (Kang et al. 1993). Therefore, it is necessary to transfer additionally of GTP cyclohydrolase-1 (GTPCH-1) gene that is the first and rate-limiting enzyme in the BH_4 biosynthetic pathway (Bencsics et al. 1996). Immortalized CNS-derived mouse NSC line C17.2 was transduced to carry tyrosine hydroxylase (TH) gene and GTP cyclohydrorylase-1 (GTPCH-1) gene for production of L-DOPA and following intra-striatal implantation behavioral improvement was seen in 6-hydroxydopamine-lesioned rats (Ryu et al. 2005). We have similarly engineered HB1.F3 human NSC line to produce L-DOPA by double transduction with human TH and GTPCH-1 genes, and following transplantation of these cells in the brain of PD rat model led to enhanced L-DOPA production *in vivo* and induced functional recovery (Kim et al. 2006).

Previous studies have reported that mouse ES cell-derived DA neurons have shown efficacy in PD animal models, whereas DA neurons from human ES cells generally show poor performance. In addition, there are considerable safety concerns for ES cells related to risk of tumor formation and neural overgrowth. More recent studies have indicated that functional human DA neurons could be generated efficiently from human ES cells and upon transplantation in rat PD models ES cell-derived DA neurons induced behavior recovery in the animals (Cho et al. 2008; Kirks et al. 2011). These studies indicate that large scale generation of DA neurons is possible from human ES cells as cellular source for cell therapy in PD patients. Human DA neurons derived from iPS cells may provide an ideal cellular source for transplantation therapy for PD. However, developing effective cell therapy approach for PD using iPS cells relies on optimizing in vitro production of iPS cell-derived DA neurons and preventing potential risk of teratoma formation in vivo. A recent study has reported generation of DA neurons from iPS cells derived from fibroblasts

and improved behavior following transplantation of the DA neurons in PD model rats (Werning et al. 2008). Although further research is still required, cell therapy based on DA neurons derived from iPS cells will probably become a promising treatment technique in the coming days.

Summary of preclinical studies of stem cell transplantation in PD animal models in rat and monkey is shown in Table 27.1.

27.3 Huntington Disease

Huntington disease (HD) is an autosomal dominant neurodegenerative disorder characterized by involuntary choreiformic movements, cognitive impairment, and emotional disturbances (Greenamyre and Shoulson 1994; Harper 1996). Despite identification of the HD gene and associated protein, the mechanisms involved in the pathogenesis of HD remain largely unknown and thus hamper effective therapeutic interventions. Transplantation of fetal human brain tissue may serve as a useful strategy in reducing neuronal damage in HD brain and a recent study has documented improvements in motor and cognition performance in HD patients following fetal cell transplantation (Bachoud-Lévi et al. 2000). This trial follows previous reports in experimental animals of HD that positive effects of fetal striatal cell transplantation to ameliorate neuronal dysfunction (Nakao and Itakura 2000) and that striatal graft tissue could integrate and survive within the progressively degenerated striatum in transgenic HD mouse model (Dunnett et al. 1998). The latter study is consistent with results obtained from HD patients indicating survival and differentiation of implanted human fetal tissue in the affected regions (Freeman et al. 2000). Cell replacement therapy using human fetal striatal grafts has shown clinical success in HD patients. However, a recent study has reported neural overgrowth of grafted tissue in a HD patient who survived 5 years post-transplantation (Keene et al. 2009). Overgrown grafts were composed of neurons and glia embedded in disorganized neuropil. This report recalls safety concerns for fetal cell grafts related to potential risk of neural overgrowth following transplantation in HD patients.

Transplantation of NSCs to replace degenerated neurons or genetically modified NSCs producing neurotrophic factors have been used to protect striatal neurons against excitotoxic insults (Bjorklund and Lindvall 2000). At present, little is known regarding whether implantation of NSCs prior to neuropathological damage could alter the progressive degeneration of striatal neurons and motor deficits that occur in HD. This question is important since genetic study of Huntington disease gene mutation (Huntington's Disease Collaborative Research Group 1993) and neuroimaging can provide details on factors involved in the progression of HD (Harris et al. 1999; Thieben et al. 2002) suggesting early intervention using brain transplantation could be effective in "pre-clinical" HD patients carrying mutant HD gene. We have investigated the effectiveness of proactive transplantation of human NSCs into rat striatum of an HD rat prior to lesion formation and demonstrated significantly

Table 27.1 Stem cell-based cell therapy in experimental Parkinson's disease models

References (number)	Animal model	Transplanted Cells	Additional treatment	Functional outcome
Kim et al. (2002)	Rat, 6-OHDA	NSC (rat)	None	Not tested
Hagell and Brundin (2002)	Rat, 6-OHDA	NPC (rat)	FGF8/SHH	Rotation↓
Takagi et al. (2005)	Monkey, MPTP	ESC (monkey)	Stromal cell (mouse) feeder	PFS-Parkinson factor score
Ryu et al. (2005)	Rat, 6-OHDA	Immortalized NSC (mouse, C17-2)	TH/GTPCH1 Gene transfer	Rotation↓
Kim et al. (2006)	Rat, 6-OHDA	Immortalized NSC (human, HB1.F3)	TH/GTPCH1 Gene transfer	Rotation↓
Yasuhara et al. (2006)	Rat, 6-OHDA	Immortalized NSC (human, HB1.F3)	NSC migration	Rotation↓
Redmond et al. (2007)	Monkey MPTP	NSC (human)	None	PFS-Parkinson factor score
Cho et al. (2008)	Rat, 6-OHDA	DA neurons from ES cells (human)	None	Rotation↓ Beam walking ↑
Werning et al. (2008)	Rat, 6-OHDA	DA neurons from ES cells (human)	WNT signal SHH	Rotation↓
Kirks et al. (2011)	Rat, 6-OHDA	DA neurons from iPS cells (human)	None	Rotation↓

ESC embryonic stem cell, *iPS cell* Induced pluripotent stem cell, *NPC* neural precursor cell, *NSC* neural stem cell, *BMSC* bone marrow mesenchymal stem cell, *6-OHDA* 6-hydroxydopamine, *MPTP* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, *TH* tyrosine hydroxylase, *GTPCH-1* GTP cyclohydrOLyrase-1

improved motor performance and increased resistance to striatal neuron damage compared with control sham injections (Ryu et al. 2004). The neuroprotection provided by the proactive transplantation of human NSCs in the rat model of HD appears to be contributed by brain-derived neurotrophic factor (BDNF) secreted by the transplanted human NSCs.

Rodents and primates with lesions of the striatum induced by excitotoxic kainic acid (KA), or quinolinic acid (QA) have been used to simulate HD in animals and to test efficacy of experimental therapeutics experiments on neural transplantation (DiFiglia 1990). Excitotoxic animal models induced by QA, which stimulates glutamate receptors, resemble the histopathologic characteristics of HD patients, were utilized for cell therapy with mouse embryonic stem cells, mouse neural stem cells, mouse bone marrow mesenchymal stem cells and primary human neural precursor cells and resulted in varying degree of clinical improvement (Kordower et al. 1997b; Armstrong et al. 2000; McBride et al. 2004; Visnyei et al. 2006; Lee et al. 2005, 2006). We have recently injected human NSCs intravenously in QA-HD model rats and demonstrated functional recovery in HD animals (Lee et al. 2005, 2006). The systemic transplantation of NSCs via intravascular route is probably the least invasive method of cell administration (Lee et al. 2006). Neural cell transplantation into striatum requires an invasive surgical technique using a stereotaxic frame. Non-invasive transplantation via intravenous routes, if it may be effective in human, is much more attractive.

Systemic administration of 3-nitropropionic acid (3-NP) in rodents leads to metabolic impairment and gradual neurodegeneration of the basal ganglia with behavioral deficits similar to those associated with HD (Beal et al. 1993; Brouillet et al. 1995), and murine and human NSCs have been transplanted in the brain of 3-NP-HD animal models (Ryu et al. 2004; Roberts et al. 2006). The compound 3-NP is a toxin which inhibits the mitochondrial enzyme succinate dehydrogenase (SDH) and tricarboxylic acid (TCA) cycle thereby interfering with the synthesis of ATP (Alston et al. 1977).

We have investigated the effectiveness of transplantation of human NSCs into adult rat striatum prior to striatal damage induced by 3-NP toxin (Ryu et al. 2004). Animals receiving intrastriatal implantation of human NSCs 1 week prior to 3-NP treatments exhibited significantly improved motor performance and increased resistance to striatal neuron damage compared with control sham injections. The neuroprotection provided by the proactive transplantation of human NSCs in the rat model of HD appears to be contributed by brain-derived neurotrophic factor (BDNF) secreted by the transplanted human NSCs. Previous studies have also demonstrated that BDNF could block neuronal injury under pathological conditions in animal models of HD (Bemelmans et al. 1999; Pérez-Navarro et al. 2000). These findings suggest that proactively transplanted human NSCs were well integrated in the striatum and supported the survival of host striatal neurons against neuronal injury.

Human NSCs derived from ESCs could provide a viable cellular source for cell and therapy in HD, since they can be expanded indefinitely and differentiate into any cell type desired. Three previous studies have shown that neurons expressing striatal markers could be induced from ESCs and brain transplantation of these ESC-derived neurons in QA-lesioned rats leads to behavioral recovery in the animals (Song et al. 2007; Aubry et al. 2008; Vasey et al. 2010).

We have recently written a review that focuses on the stem cell-based therapy for HD and investigators who wish to learn more about the subject are referred to the review article (Kim et al. 2008a, b, c). Summary of preclinical studies of stem cell transplantation in HD animal models is shown in Table 27.2.

27.4 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), known as Lou Gehric disease, is a relentlessly progressive, adult onset neurodegenerative disorder characterized by degeneration and loss of motor neurons in the cerebral cortex, brain stem and spinal cord, leading to muscle wasting and weakness, and eventually to death within 5 years after the onset of its clinical symptoms (Hudson 1990; Rowland and Shneider 2001). The proposed pathogenetic mechanisms of ALS, albeit not fully elucidated, include oxidative stress, protein aggregation, mitochondrial dysfunction, impaired axonal transport, glutamate-mediated excitotoxicity, and insufficient production of neurotrophic factors (Boillee et al. 2006). To date there is no effective treatment for patients suffering from ALS.

Recent studies have indicated that it is possible to generate motor neurons in culture from stem cells that include ESCs and NSCs (Wichterle et al. 2002; Harper et al. 2004; Miles et al. 2004; Li et al. 2005). Mouse ESC-derived motor neurons transplanted into motor neuron-injured rat spinal cord survived and extended axons into ventral root (Miles et al. 2004), and human EGCs transplanted into cerebrospinal fluid of rats with motor neuron injury migrated into spinal cord and led to improved motor function (Kerr et al. 2003). Transplantation of NSCs isolated from fetal spinal cord (Xu et al. 2006) was also effective in delaying disease progression in mouse ALS model. These cell transplantation studies have shown functional improvement in animal models of ALS.

A recent study has reported that iPSCs isolated from an ALS patient were differentiated into motoneurons (Dimos et al. 2008) and these patient-derived neurons could be an ideal cellular source for transplantation. Neurons and glia induced from patient-derived iPSCs are ideal for cell therapy as the iPSC-derived neurons are autologous, easily accessible, without immune rejection and with no ethical problem although there is safety concern of tumor formation following the cell transplantation.

The systemic transplantation of NSCs via intravascular route is probably the least invasive method of cell administration in ALS. Non-invasive transplantation via intravenous routes is much more attractive than surgical technique. Recently rat NSCs labeled with green fluorescent protein were transplanted in rat ALS model via intravenous tail vein injection and 7 days later 13 % of injected cell were found in motor cortex, hippocampus and spinal cord. No improvement in clinical symptoms was reported (Miltrecic et al. 2010).

It is unrealistic to expect the transplantation of stem cells or stem cell-derived motor neurons in ALS patients in a clinical setting replaces lost neurons, integrates into existing neural circuitry and restores motor function. Rather preventing cell death in host motor neurons via provision of neurotrophic factors by transplanted stem cells

Table 27.2 Stem cell-based cell therapy in experimental Huntington's disease models

References (number)	Animal model	Transplanted cells	Histology and lesion volume	Functional outcome
Kordower et al. (1997b)	Rat, Quinolinic acid/QA	NSC (mouse)	Intact BBB Lesion vol↓	Not tested
Armstrong et al. (2000)	Rat, QA	BMSC (mouse)	GAD+cells 0.3 % No change	No improvement
McBride et al. (2004)	Rat, QA	NPC (fetal human)	NPC migration Lesion vol↓	Cylinder test
Visnyei et al. (2006)	Rat, QA	ESC (mouse)	NeuN+cells↑ Lesion vol↓	No improvement
Lee et al. (2005)	Rat, QA	Immortalized NSC (human, HB1.F3)	NeuN+cells↑ Lesion vol↓	Circling behavior
Ryu et al. (2004)	Rat, 3-NP	Immortalized NSC (human, HB1.F3)	NeuN+cells↑ Lesion vol↓	Rotarod
Roberts et al. (2006)	Rat, 3-NP	NPC (rat)	NPC migration No change	Beam walking
Song et al. (2007)	Rat, QA	ESC-derived NSC (human)	NeuN+cells↑	Circling behavior
Aubry et al. (2008)	Rat, QA	ESC-derived NSC (human)	DARPP+neuron	Not done
Vasey et al. (2010)	Rat, QA	ESC-derived NSC (human)	Noggin-primed NSC migration	Not done

QA quinOLinic acid, *3-NP* 3-nitropropionic acid, *NSC* neural stem cell, *BMSC* bone marrow stromal cells, *NPC* neural precursor cell, *NSC* neural stem cell, *BBB* blood-brain barrier, *NeuN* neuron-specific nuclear protein

or stem cell-derived motor neurons is more realistic and achievable approach (Lindvall and Kokaia 2006). Recent studies have shown that the application of an adenoviral vector encoding glial cell line-derived growth factor (GDNF) into injured rat facial motor nucleus rescued motor neurons from cell death (Watabe et al. 2000), and human cortical progenitor cells engineered to express GDNF and transplanted into the spinal cord of ALS rats survived and released the growth factor (Kerr et al. 2003). Several recent studies have also demonstrated that delivery of vascular endothelial cell growth factor (VEGF) significantly delayed disease onset and prolonged the survival of ALS animal models (Klein et al. 2005; Azzouz et al. 2004; Zheng et al. 2004; Storkebaum et al. 2005). VEGF is one of growth factors that can be used in combination with transplanted stem cells to improve therapeutic efficiency of cellular transplantation. VEGF is an angiogenetic growth factor acting as a potent mitogen and survival factor specific to endothelial cells, and also known for neurotrophic and neuroprotective effect against brain injury. Recently we have demonstrated that in transgenic SOD1/G93A mouse model of ALS (Gurney et al. 1994) intrathecal transplantation of human NSCs over-expressing VEGF (HB1.F3.VEGF) induced functional improvement, delayed disease onset for 7 days and extended the survival of animals for 15 days (Hwang et al. 2009a). Immunohistochemical investigation of SOD1/G93A mouse spinal cord demonstrated that the transplanted human NSCs migrated into spinal cord anterior horn and differentiated into motoneurons.

More recently, we generated motoneurons from human NSCs and transplanted these cells into spinal cord of SOD1G93A ALS mouse (Kim et al. 2011). Motoneurons were generated by treatment of human NSCs encoding Olig2 bHLH transcription factor gene (HB1.F3.Olig2) with sonic hedgehog (Shh) protein. HB1.F3.Olig2 NSCs treated with Shh for 4–7 days differentiated not motoneurons expressing motoneuron-specific markers HB-9, Isl-1 and choline acetyltransferase (ChAT) but did not express OLG markers such as O4, galctocerebroside or CNPase. Control HB1.F3.Olig2 NSCs grown in the absence of Shh did not express any of the motor neuron-specific cell type markers. Intrathecal transplantation of motoneuron-committed HB1.F3.Olig2 + Shh human NSCs into L5 of spinal cord significantly delayed disease onset (9 days) and expanded the life span (22 days) of SOD1 G93A ALS mice, with two out of six mice living up to 35 days. Grafted NSCs were found within grey matter and anterior horn of the spinal cord. These results suggest that this treatment modality using genetically modified human NSCs might be of value in the treatment of ALS patients without significant adverse effects (Kim et al. 2011).

Summary of preclinical studies of stem cell transplantation in ALS animal models is shown in Table 27.3.

27.5 Alzheimer Disease

Alzheimer disease is characterized by degeneration and loss of neurons and synapses through out the brain particularly in basal fore brain, amygdala, hippocampus and cortical area. Memory and cognitive function of patients progressively decline,

Table 27.3 Stem cell-based cell therapy in experimental ALS models

References	Injury model	Transplanted cells	Additional treatment	Functional outcome
Garbuzova et al. (2002)	Mouse, SOD mutant	NT2 teratoma (human) –RA treatment	RA	Not done
Harper et al. (2004)	Rat, MN injury-sindivus virus	ESC (mouse)	RA+SHH agonist	Not done
Kerr et al. (2003)	Rat, MN injury-sindivus virus	EGC (human)	none	BBB-improvement Limb strength↑
Klein et al. (2005)	Rat, SOD mutant	NPC (human, primary)	GDNF Gene transfer	BBB-no improvement
Xu et al. (2006)	Rat, SOD mutant	NPC (human, primary)	None	BBB-improvement Extended survival
Hwang et al. (2009b)	Mouse, SOD mutant	Immortalized NSC (human HB1.F3)	VEGF Gene transfer	Rotarod, limb placement↑ Extended survival
Miltrecic et al. (2010)	Rat, SOD mutant	NSC (rat)	GFP labeled	Not done
Kim et al. (2011)	Mouse, SOD mutant	Immortalized NSC (human HB1.F3)	Olig2 Gene transfer Shh treatment	Rotarod, limb placement↑ Extended survival

SOD superoxide dismutase, *MN* motor neuron, *NPC* neural precursor cell, *ESC* embryonic stem cell, *EGC* embryonic germinal stem cell, *NSC* neural stem cell, *GDNF* glial cell line-derived neurotrophic factor, *RA* retinoic acid, *SHH* sonic hedgehog, *VEGF* vascular endothelial growth factor, *GFP* green fluorescent protein

patients become demented and die prematurely (Whitehouse et al. 1981; Bartus et al. 1982; Coyle et al. 1983). No effective treatment is currently available except for acetylcholinesterase inhibitors which augment cholinergic function but is not curative and only a temporary measure.

As for the pathogenesis of AD, the amyloid cascade hypothesis postulates that memory deficits are caused by increased levels of both soluble and insoluble amyloid ß (Aß) peptides, which are derived from the larger amyloid precursor protein (APP) sequential proteolytic processing (Whitehouse et al. 1981; Bartus et al. 1982; Coyle et al. 1983; Hardy and Selkoe 2002). Recent study has reported that treatment of PDAPP mice, a transgenic mouse model of AD, with anti-Aß antibody completely restored hippocampal acetylcholine release and high-affinity choline uptake and improved habituation learning (Bales et al. 2006). Based on the study, a clinical trial in AD patients is underway in the US.

Chronically decreasing Aß levels in brain has been suggested as a possible therapeutic approach for AD, and several experimental evidence indicate that proteinases such as neprilysin (Iwata et al. 2001), insulin degrading enzyme (Farris et al. 2003; Miller et al. 2003), plasmin (Melchor et al. 2003) and cathepsin B (Mueller-Steiner et al. 2006) could be used as therapeutic agents to reduce Aß levels in AD brain. Recent studies have shown that intracerebral injection of a lentivirus vector expressing human neprilysin in transgenic mouse models of amyloidosis reduced Aß deposits in the brain and blocked neurodegeneration in the frontal cortex and hippocampus (Marr et al. 2003), and that intracerebrally injected fibroblasts overexpressing human neprilysin gene were found to significantly reduce amyloid plaque burden in the brain of Aß transgenic mice (Hemming et al. 2007). These studies support the use of Aß-degrading proteases as a tool to therapeutically lower Aß levels and encourage further investigation of ex vivo delivery of protease genes using human NSCs for the treatment of AD. We have recently generated a human NSC line encoding human neprylysin gene, transplanted these cells in lateral ventricle of AD transgenic mouse brain, and results are expected some time later.

Earlier studies have indicated that nerve growth factor (NGF) prevent neuronal death and improve memory in animal models of aging, excitotoxicity and amyloid toxicity (Hefti 1986; Fischer et al. 1987; Tuszynski et al. 1990; Emerich et al. 1994; Tuszynski 2002), and could be used for treating neuronal degeneration and cell death in AD brain. However, delivery of NGF into the brain is not possible via peripheral administration. Because of its size and polarity NGF does not cross the blood brain barrier. In order to overcome this difficulty, gene therapy approach could be adopted. Using *ex vivo* gene therapy approach via NGF encoding cells, NGF can be administered directly to the brain and diffuse for distance of 2–5 mm (Tuszynski et al. 1990). A phase 1 clinical trial of *ex vivo* NGF gene delivery was performed in eight mild AD patients by implanting autologous fibroblasts genetically modified to express human NGF into the forebrain. After mean follow-up of 22 months in 6 subjects, long-term adverse effects were not found. Evaluation by MMSE and AD A SCS suggested improvement in the rate of cognitive decline. Serial PET scans showed significant increases in cortical fluorodeoxyglucose after treatment (Tuszynski et al. 2005). Since fibroblasts are known for their immobility

following brain transplantation (Kang et al. 1993), NSCs with high migratory capacity and pathology-tropic property (Flax et al. 1998; Kim 2004; Lee et al. 2007a; Kim and deVellis 2009) could be used in place of fibroblasts to deliver NGF into the AD brain. In learning deficit AD model rats induced by okadaic acid injection, transplantation of rat NSCs infected with adenovirus-NGF produced improvement in cognitive performance (Wu et al. 2008).

In a recent study, we used human NSCs in place of rodent NSCs or human fibroblasts to deliver NGF in ibotenic acid-induced learning deficit rats. Intrahippocampal injection of ibotenic acid caused severe neuronal loss, resulting in learning and memory deficit (Lee et al. 2012). NGF protein released by HB1.F3. NGF human NSCs in culture media is tenfold over the control F3 naive NSCs at 1.2 μg/10^6 cells/day. Intra-hippocampal transplantation of HB1.F3.NGF cells was found to express NGF and fully improved the learning and memory function of ibotenic acid-challenged animals. Transplanted HB1.F3.NGF human NSCs were found all over the brain and differentiated into neurons and astrocytes (Lee et al. 2012). In another study, brain derived neurotrophic factor (BDNF), a member of neurotrophin family, secreted by transplanted mouse NSCs was responsible in enhancing cognitive function in triple transgenic mice that express pathogenic forms of myloid precursor protein, presenilin and tau. In these animals cognition was improved without altering Aβ or tau pathology (Blurton-Jones et al. 2009). In other studies in experimental rats with nucleus basalis of meynert (NBM) lesion induced by ibotenic acid, transplantation of mouse or rat neural precursor cells (NPCs) promoted behavioral recovery (Wang et al. 2006; Moghadam et al. 2009).

In AD patients, dysfunction of the presynaptic cholinergic system is one of the causes of cognitive disorders where decreased activity of choline acetyltransferase (ChAT), which is responsible for acetylcholine (ACh) synthesis, is observed (Terry and Buccafusco 2003). To date, AD therapy has largely been based on small molecules designed to increase ACh concentration by inhibiting acetylcholinesterase (Musiał et al. 2007). Since therapies with these drugs is only palliative without potential protection against progressive tissue destruction, there is a need for effective therapies for patients with AD, and stem cell-based therapeutic approaches targeting AD should fulfill this requirement. We have recently generated a human neural stem cell (NSC) line over-expressing human choline acetyltransferase (ChAT) gens and these HB1.F3.ChAT NSCs were transplanted into the brain of rat Alzheimer disease (AD) model which was generated by intra-hippocampal injection of kainic acid (KA) in CA3 region which results in severe neuronal loss and profound learning and memory deficit. Intraventricular transplantation of HB1.F3.ChAT human NSCs fully restored learning and memory (Park et al. 2012a). Similarly HB1.F3.ChAT human NSCs were transplanted in AD model rats generated by application of ethylcholine mustard aziridinium ion (AF64A) that specifically denatures chOLinergic nerves and thereby leads to memory deficit as a salient feature of AD (Yamazaki et al. 1991). Transplantation of HB1.F3.ChAT human NSCs in AF64A-treated mice fully restored the learning and memory function of AF64A animals (Park et al. 2012b).

Summary of preclinical studies of stem cell-based cell therapy in AD animal models is shown in Table 27.4.

Table 27.4 Stem cell-based cell therapy in experimental Alzheimer's disease models

References (number)	Animal model	Transplanted cells	Special feature	Outcome
Wang et al. (2006)	Mouse NBM lesion Ibotenic acid -	ESC-derived neurosphere (mouse)	ChAT+cells↑	Working memory↑
Wu et al. (2008)	Rat Forebrain Okadaic acid	NSC (rat)	NGF(human) Gene transfer	Memory↑
Moghadam et al. (2009)	Rat NBM lesion Ibotenic acid	ESC-derived NPC (mouse)	Shh-primed	Water maze↑ Spatial probe↑
Blurton-Jones et al. (2009)	Mouse 3X TG-AD	NSC (mouse)	BDNF-mediated effect	Working memory↑
Park et al. (2012a)	Rat Hippocampus Kainic acid	Immortalized NSC (human, HB1.F3)	ChAT (human) Gene transfer	Water maze↑ Spatial probe↑
Park et al. (2012b)	Rat NBM lesion AF64A toxin	Immortalized NSC (human, HB1.F3)	ChAT (human) Gene transfer	Water maze↑ Spatial probe↑
Lee et al. (2012)	Mouse Hippocampus Ibotenic acid	Immortalized NSC (human, HB1.F3)	NGF (human) Gene transfer	Water maze↑ Spatial probe↑

NBM Nucleus basalis of Meynert, *NSC* neural stem cell, *ESC* embryonic stem cell, *NPC* neural precursor cell, *NGF* nerve growth factor, *BDNF* brain derived growth factor, *SHH* sonic hedgehog protein, *ChAT* choline acetyltransferase, *3XTG* triple transgenic/APP-presenilin-tau

27.6 Multiple Sclerosis

In multiple sclerosis (MS), Oligodendrocytes (OLGs) and myelin are destroyed by inflammation-mediated mechanism (McFarlin and McFarland 1982; Ebers 1988). Although recent advance in treatment using immune moderators such as interferon-β has improved clinical outcome in some patients, functional recovery in most of MS patients is not achieved (Paty and Ebers 1998). Therefore, there is substantial need for effective therapies for MS patients.

Previous studies have reported that OLGs or OLG progenitor cells isolated from mouse or rat brain were transplanted in the brain of dysmyelination mutants or chemically induced demyelination lesions in rats and induced remyeliation in previously dysmyelinated or demyelinated lesion sites (Franklin and Blakemore 1997; Espinosa de los Monteros et al. 1997, 2001; Learish et al. 1999; Zhang et al. 1999; Ben-Hur et al. 2003). Experimental animal models for MS used in transplantation studies include shiverer dymyelination mutant mouse, demyelination lesions induced by ethidium bromide, experimental allergic encephalitis (EAE) or mouse hepatitis virus. Therapeutic approach with myelinating glia could be applied in MS patients by transplantation of human OLGs into demylination lesions. Intact embryonic human brain fragments or OLG progenitors isolated from fetal human brain have been placed in shiverer mouse brain, a mouse neurological mutant with defect in myelin basic protein gene, and remyelination was confirmed (Lachapelle et al. 1983; Gumpel et al. 1987; Seilhean et al. 1996; Windrem et al. 2004). Transplantation of human OLGs in MS patients to achieve remyelination of previously demyelinated axons, however, has not been undertaken to date. This therapeutic approach of transplantation of human OLGs or OLG progenitors derived from fetal brain is not widely acceptable because of moral, religious and logistic problems associated with tissue collection of human embryonic/fetal brain. In addition, the outcome of graft is not predictable since the implanted embryonic/fetal tissues contain mixed population of neurons, glial cells and CNS progenitor cells, and less than 10 % of cell population for graft expressed O4, a marker for young OLG (Gumpel et al. 1987). This difficulty can be circumvented by utilization of OLGs or OLG progenitor cells derived from human ES cells or NSCs. Recent studies have reported that OLGs could be generated from mouse and human ES cells (Brüstle et al. 1999; Liu et al. 2000; Glaser et al. 2005; Nistor et al. 2005), bone marrow mesenchymal stem cells (Akiyama et al. 2002) or immortalized mouse NSCs (Yandava et al. 1999). In a mouse EAE, systemically injected mouse neural precursor cells (NPCs) selectively enter the inflamed CNS in EAE model and induce apoptosis of blood-borne CNS-infiltrating encephalitogenic T cells, thus protecting against chronic neural tissue loss. NPCs display immune-like functions that promote neuroprotection in the CN (Pluchino et al. 2005). Similarly human ESC-derived NPCs transplanted into the brain ventricles of EAE mouse reduced clinical signs of EAE and transplanted NPCs were found in the white matter. These results indicate that NPCs act as immune-like cells in the CNS (Aharonowiz et al. 2008).

OLGs could also be generated from stable established cell lines of human NSC and used as cell source of transplantation. Previously we have produced immortalized cell lines of human NSC from human embryonic telencephalon using a retroviral vector encoding myc oncogene (Kim 2004; Lee et al. 2007a; Kim et al. 2008c). This human NSC line, HB1.F3 (F3), can be genetically engineered to express foreign transgenes, and following transplantation into brain of animal models of PD, HD and stroke, F3 NSCs survived, differentiate into neurons and astrocytes, and reversed functional deficits (Kim 2004; Lee et al. 2007a; Kim and deVellis 2009). Most recently we were successful in producing a new F3.Olig2 human NSC line by transduction of F3 with a retroviral vector encoding Olig2 bHLH transcription factor gene. Olig2 is a member of Olig bHLH transcription factor family and plays a crucial role in generation of OLGs and ensueing myelination in the CNS, and also a key factor for generation of spinal motoneurons (Lu et al. 2000; Zhou et al. 2000; Takebayashi et al. 2000; Copray et al. 2006). F3.Olig2 human NSCs, we have generated, express cell type specific markers for OLG progenitors (PDGFRα and NG2), and also cell type specific markers for OLG (O4, galactocerebroside and CNP). F3.Olig2 NSCs were transplanted in contused rat spinal cord lesion site and at 7 weeks post-transplantation grafted NSCs were found in the white matter and differentiated into mature OLGs. Animals with F3.Olig2 grafts showed an improvement in hindlimbs locomotion (Hwang et al. 2009a, b).

Following transplantation into the shiverer mouse brain, a mouse neurological mutant with congenital dysmyelination, an extensive myelination was demonstrated (unpublished data). We expect this successful pre-clinical study could lead to cell-based therapy in MS patients, with provision of unlimited number of human OLG/OLG progenitor cells for transplantation from this human cell line.

No treatments are currently available that slow, stop, or reverse disease progression in established MS. Currently a phase II multicenter study of autologous MSC transplantation in secondary progressive MS is ongoing and the results of clinical outcome is expected 9n a year or two (Connick et al. 2011).

Summary of preclinical studies of stem cell transplantation in MS animal models is shown in Table 27.5.

27.7 Stroke

Stroke represents the second highest among the causes of death in East Asia including China, Japan and Korea, and third highest in US. There are two major types pf stroke and they are ischemia and intracerebral hemorrhage (ICH). Ischemic stroke caused by abrupt and near-total interruption of cerebral blood flow, produces ischemic changes in the striatum and cortex, leading to a long-term sensorimotor deficit. The major cause of ICH is hypertension and less common causes include trauma, infections, tumors, blood clotting deficiencies, and abnormalities in blood vessels such as arteriovenous malformations. Once damage from a stroke occurred, little can be done to restore premorbid functions, and although numerous neuroprotective

Table 27.5 Stem cell-based cell therapy in experimental multiple sclerosis models

References (number)	Animal model	Transplanted cells	Special feature	Outcome
Espinosa de los Monteros et al. (1997)	Rat, md mutant -	OPC (rat, CG4)	Beads label	Remyelination
Yandava et al. (1999)	Mouse, shiverer mutant	Immortalized NSC (mouse, C17-2)	None	Remyelination
Liu et al. (2000)	Rat, sp cord Ethidium bromide lesion	ESC (mouse)	FGF8/SHH	Remyelination
Akiyama et al. (2002)	Rat, sp cord contusion	BMSC (rat)	None	Remyelination
Glaser et al. (2005)	Rat, md mutant -	ESC (mouse)	None	Remyelination
Nistor et al. (2005)	Rat, sp cord contusion	ESC (human)	None	Remyelination
Copray et al. (2006)	Mouse, cuprizone	NPC (mouse) 20 % OLG	Olig2 bHLH Gene transfer (transient)	Remyelination
Hwang et al. (2009a, b)	Mouse, sp cord contusion	Immortalized NSC (human, HB1.F3)	Olig2 bHLH Gene transfer	Remyelination

OPC Oligodendrocyte progenitor cell, *NSC* neural stem cell, *ESC* embryonic stem cell, *BMSC* bone marrow mesenchymal stem cell, *NPC* neural precursor cell, *SHH* sonic hedgehog protein, *OLG* oligodendrocyte, *bHLH* basic helix loop helix transcription factor

agents have been clinically tried, no specific agents replaced the lost neurons, improved the deteriorated functions, and reduced the long-term sequelae (Marshall and Thomas 1988, 130). There are numerous previous reports of stem cell transplantation in the stroke animal models (Savitz et al. 2002), and various cellular sources such as rodent bone marrow MSCs (Sinden et al. 1997; Chen et al. 2001, 2003; Zhao et al. 2002; Modo et al. 2002), mouse neural precursor cells (Veizovic et al. 2001), human umbilical cord blood cells (Chen et al. 2001), human bone marrow MSCs (Kim et al. 2008b; Cho et al. 2010; Ding et al. 2011), human teratocarcinoma-derived neurons (Borlongan et al. 1998; Saporta et al. 1999) and ESC- or iPSC-derived NSCs (Daadi et al. 2010; Jin et al. 2010; Kawai et al. 2010) were grafted into the ischemic rodent brain, and reduced the neurological deficits. An ealier study has reported that in human with ischemic infarct, intracerebral implantation of human teratocarcinoma NT2-derived neurons has resulted in functional improvement (Kondziolka et al. 2000).

Neural stem cells (NSCs) could be isolated from embryonic, fetal or adult CNS tissues of mammals including human. NSCs and neurons could also be derived from ESCs or iPSCs and transplanted in animal models of stroke inducing functional recovery in the animals (Daadi et al. 2010; Jin et al. 2010; Seminatore et al. 2010; Kawai et al. 2010). However, risk of tumor formation is a major obstacle to cell therapy based on human ESC- or iPSC-derived cells. Human neural progenitor cells (NPCs) were isolated from four differentiation stage of ESCs and transplanted into rats with MCA lesions, and the tumorigenesis is linked to NPCs derived from later differentiation stages of ESCs (Seminatore et al. 2010). In another study, transplantation of iPS-derived cells into rat ischemic brain resulted in tumor formation at 4 weeks pos-transplantation (Kawai et al. 2010). ESC- and iPSC-derived cells have a promising potential to provide neurons and glia for the cell therapy in stroke. However proper and strict control of tumorigenesis has to be achieved before ESC- or iPSC-based cell therapy becomes a realistic clinical strategy.

We have previously investigated whether conditionally immortalized human NSCs could selectively migrate into lesioned brain sites, differentiate into new neurons and/or glia, and improve the functional deficits in rat stroke models of focal ischemia (Chu et al. 2003, 2005) and cerebral hemorrhage (Jeong et al. 2003; Lee et al. 2007a, b, 2008, 2009a, b, 2010a, b). NSCs can circumvent blood-brain barrier and migrate to the specific pathologic areas of brain with tropism. We introduced immortalized human NSCs intravenously via tail veins or into lesion site and NSCs migrated into the adult rat/mouse brain with transient focal cerebral ischemia or with cerebral hemorrhage. Transplanted human NSCs migrated to the lesion site, differentiated into neurons and astrocytes, and a large number of the grafted human NSCs survived in the lesion sites for up to 12 weeks. Functional improvement was observed in the transplanted animals compared with non-grafted controls on rotarod and turning-in-an-alley tests. Transplantation of NSCs overexpressing neurotrophic factors such as vascular endothelial growth factor (VEGF), glial cell line-derived neurotrophic factor (GDNF) or brain derived growth factor (BDNF) induced good survival and neuroprotection of both host neurons and grafted NSCs in the lesion site and promoted functional improvement in the ICH model animals (Lee et al. 2007a, b, 2009a, b, 2010a, b).

Bone marrow-derived mesenchymal stem cells (MSCs) have been also engrafted in animal models of stroke and found to survive and ameliorate functional deficits in the animals (rodent MSCs – Zhao et al. 2002; Chen et al. 2003, 2009; Modo et al. 2002; Guzman et al. 2008; human MSCs – Kurozumi et al. 2004; Kim et al. 2008b; Cho et al. 2010; Pendharkar et al. 2010; Ding et al. 2011), raising the possibility of therapeutic potential of MSCs for repair of damaged brain following ischemic or hemorrhagic injury. It is worthy to note that transplantation of MSCs genetically modified to express neurotrophic molecules such as erythropoietin (Cho et al. 2010), or NGF and Noggin (Ding et al. 2011) in stroke model animals induced higher number of surviving cells and improved function. In another study, transplantation of human MSCs expressing Neurogenin1 (Ngn1), a proneural gene that directs neuronal differentiation of neural progenitor cells, in the rat ischemic stroke model improved motor functions as compared with control naive MSCs. This study indicates that the neurons induced from MSCs are far better cell source for cell therapy in stroke (Kim et al. 2008b).

We have previously generated a stably immortalized human MSC cell line derived from fetal bone marrow, and following brain transplantation in ICH mice, human MSCs were found to integrate into host brain, differentiate into neurons and astrocytes, and induce functional recovery in the animals (Nagai et al. 2007).

Only small number of clinical trials using MSCs were performed to date and one study on long-term safety and efficacy of intravenous MSC transplantation in a large population of stroke patients (52 patients) reported that the MSC therapy improved clinical outcome in 16 patients who received autologous MSCs (Lee et al. 2011). In another study, autologous MSCs derived from stroke patients were expanded in human serum and delivered intravenously in 12 patients. There were no CNS tumors, abnormal cell growths or neurological deterioration following MSC infusion. Mean lesion volume as assessed by MRI was reduced by >20 % at 1 week post-cell infusion. This study provides evidence indicating the feasibility and safety of delivery of a relatively large dose of autologous MSCs into stroke patients (Honmou et al. 2011).

Summary of preclinical studies of stem cell transplantation in stroke animal models is shown in Table 27.6.

27.8 Perspectives

There are a number of issues to be clarified before adoption of stem cells for cell replacement therapy and gene therapy is widely accepted in clinical medicine such as which type of stem cells are most suitable for cell replacement therapy in patients with neurological disorders or brain injury, and safety issues related to the risk of tumorigenesis by grafted stem cells. Since neurons could be derived not only from NSCs, but also from ESCs, EGCs, bone marrow MSCs, umbilical cord blood hematopoietic stem cells and even from iPS cells generated from adult somatic cells, the most pressing question is which cells are best suited for cell replacement

Table 27.6 Stem cell-based cell therapy in experimental stroke models

References (number)	Animal model	Transplanted cells	Transplantation route	Functional outcome
Zhao et al. (2002)	Rat, MCA occulusion	BMSC (rat)		Limb placement↑ Tactile stimulation
Chen et al. (2003)	Rat, MCA occulusion	BMSC (rat)	Intravenous	Rotarod↑ Adhesive removal
Modo et al. (2002)	Rat MCA occulusion	BMSC (rat)	–	Rotarod↑ Adhesive removal
Veizovic et al. (2001)	Rat, MCA occulusion	Immortalized NPC (rat)		Roarod↑ Water maze↑
Borlongan et al. (1998)	Rat, MCA occulusion	NT2 Teratoma (human) RA treatment	Intracerebral	Rotarod↑ Neurol score↑
Chu et al. (2003)	Rat, MCA occulusion	Immortalized NSC (human, HB1.F3)	Intravenous	Roarod↑ Neurol score↑
Jeong et al. (2003)	Rat, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intravenous	Roarod↑ Neurol score↑
Lee et al. (2005)	Rat, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intravenous	Roarod↑ Neurol score↑
Chu et al. (2005)	Rat, MCA occulusion	Immortalized NSC (human, HB1.F3)	Intravenous	Roarod↑ Neurol score↑
Lee et al. (2007a)	Rat, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intracerebral	Roarod↑ Neurol score↑
Kurozumi et al. (2004)	Rat, MCA occulusion	BMSC	Intracerebral BDNF gene Transfer (Adeno)	Limb placement↑ Treadmil↑
Nagai et al. (2007)	Rat, ICH collagenase	Immortalized BMSC (human B10)	Intracerebral	Roarod↑ Limb placement↑

(continued)

Table 27.6 (continued)

References (number)	Animal model	Transplanted cells	Transplantation route	Functional outcome
Lee et al. (2007b)	Mouse, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intracerebral VEGF gene transfer	Roarod↑ Neurol score↑
Lee et al. (2009a)	Mouse, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intracerebral GDNF gene transfer	Roarod↑ Limb placement↑
Lee et al. (2009b)	Mouse, MCA occulusion	Immortalized NSC (human, HB1.F3)	Intracerebral Akt1 gene Transfer	Roarod↑ Limb placement↑
Lee et al. (2010a, b)	Mouse, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intracerebral BDNF gene transfer	Roarod↑ Limb placement↑
Daadi et al. (2010)	Rat, MCA occulusion	ESC-derived NSC (human)	Intracerebral	Neurol score↑
Jin et al. (2010)	Rat, MCA occulusion	ESC-derived NSC (human)	Intracerebral	Neurol score↑
Kim et al. (2008b)	Rat, MCA occulusion	MSC (human)	Intracerebral Ngn1 gene transfer	Roarod↑ Limb placement↑
Ding et al. (2011)	Rat, MCA occulusion	MSC (human)	Intracerebral NGF/Noggin gene (adeno)	Neurol score↑
Cho et al. 2010	Rat, MCA occulusion	MSC (human)	Intracerebral EPO gene transfer	Neurol score↑
Lee et al. (2011)	Human stroke patients	MSC (human Autologous)	Intravascular	Neurol score↑ (16 out of 52 patients)
Honmou et al. (2011)	Human Stroke patients	MSC (human Autologous)	Intravascular	Clinical outcome not available (12 patients)

MCA middle cerebral artery, *ICH* intracerebral hemorrhage, *BMSC* bone marrow mesenchymal stem cell, *NPC* neural precursor cell, *NSC* neural stem cell, *VEGF* vascular endothelial growth factor, *GDNF* glial cell line derived neurotrophic factor, *BDNF* brain derived neurotrophic factor, *NGF* nerve growth factor, *EPO* erythropoietin

therapy. Since the presence of NSCs in adult CNS is known, it is only a matter of time before neurons and glial cells are cultured from adult CNS tissue samples. There are ongoing debates as to why oocytes, embryonic or fetal materials should be used to generate stem cells when stem cells could be isolated from adult tissues. However, most of research up to now indicates that embryonic or fetal stem cells are significantly more versatile and plastic than adult counterparts.

Previous studies have demonstrated that ESC- or NSC-derived neurons or glial cells could be renewable cell source in cell based therapy for patients suffering from neurological diseases including PD, HD, ALS, AD, MS, stroke and spinal cord injury, however, there exist serious caveats that limit the use of stem cell-derived neurons or glial cells for the purpose. The considerations include (i) the long-term survival and phenotype stability of stem cell-derived neurons or glial cells in the graft following transplantation are not favorable as earlier studies have demonstrated, (ii) highly purified populations of neuronal cell type derived from ESCs or NSCs may contain other neuronal or glial cell types that might produce unpredictable interactions among grafted cells or with host neurons, and (iii) a small number of ESCs or iPSCs that escaped differentiation and selection processes might expand and form tumor in the graft site following transplantation.

Continuously dividing immortalized cell lines of human NSCs as generated by introduction of oncogenes have advantageous features for cell replacement therapy and gene therapy and the features include that human NSCs are homogeneous since they were generated from a single clone, can be expanded to large numbers in vitro, and stable expression of therapeutic genes can be achieved readily. Immortalized human NSCs have emerged as highly effective source of cells for genetic manipulation and gene transfer into the CNS ex vivo and once transplanted into damaged brain they survive well, integrate into host tissues and differentiate into both neurons and glial cells. It is known that both extrinsic and heritable intrinsic signals play important roles in generating cellular diversity in the CNS. By introducing relevant signal molecules or regulatory genes into the human stem cell line, it is now possible to obtain a large number of selected populations of neurons or glial cells from continuously growing human NSCs. Further studies are needed in order to identify the signals for proliferation, differentiation and integration of NSCs and determine favorable conditions of host brain environment for implanted NSCs to survive, prosper and restore the damaged brain.

References

Agid Y (1991) Parkinson's disease: pathophysiology. Lancet 337:1321–1324

Aharonowiz M, Einstein O, Fainstein N, Lassmann H, Reubinoff B, Ben-Hur T (2008) Neuroprotective effect of transplanted human embryonic stem cell-derived neural precursors in an animal model of multiple sclerosis. PLoS One 3:e3145

Akiyama Y, Radke C, Kocsis JD (2002) Remyelination of rat spinal cord by implantation of identified bone marrow stromal cells. J Neurosci 22:6623–6630

Alston TA, Mela L, Bright HJ (1977) 3-Nitropropionate, the toxic substrate of indigofera, si a suicide inactivator of succinate dehydrogenase. Proc Natl Acad Sci USA 74:3767–3771

Anton R, Kordower JH, Maidment NT, Manaster JS, Kane DJ, Rabizadeh S, Schueller SB, Yang J, Rabizadeh S, Edwards RH (1994) Neural-targeted gene therapy for rodent and primate hemiparkinsonism. Exp Neurol 127:207–218

Armstrong RJ, Watts C, Svendsen CN, Dunnett SB, Rosser AE (2000) Survival, neuronal differentiation, and fiber outgrowth of propagated human neural precursor grafts in an animal model of Huntington's disease. Cell Transplant 9:55–64

Aubry L, Bugi A, Lefort N, Rousseau F, Peschanski M, Perrier AL (2008) Striatal progenitors derived from ES cells mature into DARPP32 neurons in vitro and in quinolinic acid-lesioned rats. Proc Natl Acad Sci USA 105:16707–16712

Azzouz M, Ralph GS, Storkebaum E, Walmsley LE, Mitrophanous KA, Kingsman SM, Carmeliet P, Mazarakis ND (2004) VEGF delivery with retrogradely transported lentivector prOLongs survival in a mouse ALS model. Nature 429:413–417

Bachoud-Lévi AC, Rémy P, Nguyen JP, Brugières P, Lefaucheur JP, Bourdet C, Baudic S, Gaura V, Maison P, Haddad B, Boissé MF, Grandmougin T, Jény R, BartOLomeo P, Dalla Barba G, Degos JD, Lisovoski F, Ergis AM, Pailhous E, Cesaro P, Hantraye P, Peschanski M (2000) Motor and cognitive improvements in patients with Huntington's disease after neural transplnatation. Lancet 356:1975–1979

Bales KR, Tzavara ET, Wu S, Wade MR, Bymaster FP, Paul SM, Nomikos GG (2006) Cholinergc dysfunction in a mouse model of Alzheimer disease is reversed by an anti-Aβ antibody. J Clin Invest 116:825–832

Bartus R, Dean RL, Beer B, Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408–411

Beal MF, Brouillet E, Jenkins BG, Ferrante RJ, Kowall NW, Miller JM, Storey E, Srivastava R, Rosen BR, Hyman BT (1993) Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. J Neurosci 13:4181–4192

Bemelmans AP, Horellou P, Pradier L, Brunet I, Colin P, Mallet J (1999) Brain-derived neurotrophic fator-mediated protection of striatal neurons in an excitotoxic rat model of Huntington's disease, as demonstrated by adenoviral gene transfer. Hum Gene Ther 10:2987–2997

Bencsics C, Wachtel SR, Milstien S, Hatakeyama K, Becker JB, Kang UJ (1996) Double transduction with GTP cyclohydrolase1 and tyrosine hydroxylase is necessary for spontaneous synthesis of L-DOPA by primary fibroblasts. J Neurosci 16:4449–4456

Ben-Hur T, Einstein O, Mizrachi-KOL R, Ben-Menachem O, Reinhartz E, Karussis D, Abramsky O (2003) Transplanted multipotential neural progenitor cells migrate into the inflamed white matter in response to experimental allergic encephalitis. Glia 41:73–80

Bjorklund A, Lindvall O (2000) Cell replacement therapies for central nervous system disorders. Nat Neurosci 3:537–544

Björklund A, Stenevi U (1979) Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. Brain Res 177:555–560

Blurton-Jones M, Kitazawa M, Martinez-Coria H, Castello NA, Muller FJ, Loring JF, Yamasaki TR, Poon W, Green KN, LaFerla FM (2009) Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. Proc Natl Acad Sci USA 106:13594–13598

Boillee S, Van de Velde C, Cleveland DW (2006) ALS: a disease of motor neurons and their nonneuronal neighbors. Neuron 52:39–59

Borlongan CV, Tajima Y, Trojanowski JQ, Lee VM, Sanberg PR (1998) Transplantation of cryopreserved human embryonic carcinoma-derived neurons (NT2N cells) promotes functional recovery in ischemic rats. Exp Neurol 149:310–321

Brouillet E, Hantraye P, Ferrante RJ, Dolan R, Leroy-Willig A, Kowall NW, Beal MF (1995) Chronic mitochondrial energy impairment produces selective striatal degeneration and abnormal movements in primates. Proc Nat Acad Sci USA 92:7105–7109

Brustle O, McKay RG (1996) Neuronal progenotors as tools for cell replacement in the nervous system. Curr Opin Neurobiol 6:688–695

Brüstle O, Jones KN, Learish RD, Karram K, Choudhary K, Wiestler OD, Duncan ID, McKay RD (1999) Embryonic stem cell-derived glial precursors: a source for myelinating transplants. Science 285:754–756

Chen J, Sanberg PR, Li Y, Wang L, Lu M, Willing AE, Sanchez-Ramos J, Chopp M (2001) Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. Stroke 32:2682–2688

Chen J, Li Y, Katakowski M, Chen X, Wang L, Lu D, Lu M, Gautam SC, Chopp M (2003) Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. J Neurosci Res 73:778–786

Chen B, Gao XQ, Yang CX, Tan SK, Sun ZL, Yan NH, Pang YG, Yuan M, Chen GJ, Xu GT, Zhang K, Yuan QL (2009) Neuroprotective effect of grafting GDNF gene-modified neural stem cells on cerebral ischemia in rats. Brain Res 1284:1–11

Cho MS, Lee YE, Kim JY, Chung S, Cho YH, Kim D, Kang S, Lee H, Kim M, Kim J, Leem JW, Oh SK, Choi YM, Hwang D, Chang JW, Kim D (2008) Highly efficient and large scale generation of functional dopamine neurons from human embryonic stem cells. Proc Natl Acad Sci USA 105:3387–3392

Cho GW, Koh SH, Kim MH, Yoo AR, Noh MY, Oh S, Kim SH (2010) Neuroprotective effect of erythropoietin-transduced human mesenchymal stromal cells in an animal model of ischemic stroke. Brain Res 1353:1–13

Chu K, Kim M, Jeong SW, Kim SU, Yoon BW (2003) Human neural stem cells can migrate, differentiate and integrate after intravenous transplantation in adult rats with transient forebrain ischemia. Neuroci Lett 343:637–643

Chu K, Park KI, Lee ST, Jung KH, Ko SY, Kang L, Sinn DI, Lee YS, Kim SU, Kim M, Roh JK (2005) Combined treatment of vascular endothelial growth factor and human neural stem cells in experimental focal cerebral ischemia. Neurosci Res 53:384–390

Chung S, Sonntag KC, Andersson T, Bjorklund LM, Park JJ, Kim DW, Kang UJ, Isacson O, Kim KS (2002) Genetic engineering of mouse embryonic stem cells by Nurr1 enhances differentiation and maturation into dopaminergic neurons. Eur J Neurosci 16:1829–1838

Connick P, Kolappan M, Patani R, Scott M, Crawley C, He X, Richardson K, Barber K, Webber D, Claudia A, Wheeler-Kingshott C, Tozer D, Samson D, David L, Thomas D, Du M, Luan S, Michell A, Daniel R, Altmann D, Thompson A, Miller D, Compston A, Chandran S (2011) The mesenchymal stem cells in multiple sclerosis (MSCIMS) trial protocol and baseline cohort characteristics: an open-label pre-test: post-test study with blinded outcome assessments. Trials 12:62

Copray S, Balasubramaniyan V, Levenga J, de Bruijn J, Liem R, Boddeke E (2006) Olig2 overexpression induces the in vitro differentiation of neural stem cells into mature Oligodendrocytes. Stem Cells 24:1001–1010

Coyle JT, Price DL, DeLong MR (1983) Alzheimer's disease: a disorder of cortical cholinergic innervation. Science 219:1184–1190

Daadi MM, Davis AS, Arac A, Li Z, Maag AL, Bhatnagar R, Jiang K, Sun G, Wu JC, Steinberg GK (2010) Human neural stem cell grafts modify microglial response and enhance axonal sprouting in neonatal hypoxic-ischemic brain injury. Stroke 41(516–523):2010

DiFiglia M (1990) Excitotoxic injury of the neostriatum: a model for Huntington's disease. Trends Neurosci 13:286–289

Dimos JT, RodOLfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, Croft GF, Saphier G, Leibel R, Goland R, Wichterle H, Henderson CE, Eggan K (2008) Induce pluripotent stem cells generated from patient with ALS can be differentiated into motor neurons. Science 321:1218–1221

Ding J, Cheng Y, Gao S, Chen J (2011) Effects of nerve growth factor and Noggin-modified bone marrow stromal cells on stroke in rats. J Neurosci Res 89:222–230

Dunnett SB, Bjorklund A (1999) Prospects for new restorative and neuroprotective treatments in Parkinson's disease. Nature 399:A32–A39

Dunnett SB, Carter RJ, Watts C, Torres EM, Mahal A, Mangiarini L, Bates G, Morton AJ (1998) Striatal transplantation in a transgenic mouse model of Huntington's disease. Exp Neurol 154:31–40

During MJ, Naegele JR, O'Malley KL, Geller AI (1994) Long-term behavioral recovery in parkinsonian rats by an HSV vector expressing tyrosine hydroxylase. Science 266: 1399–1403

Ebers GC (1988) Multiple scelrosis and other demyelinating diseases. In: Asbury A, McKhann G, McDonald W (eds) Diseases of the nervous system. WB Saunders, Philadelphia, pp 1268–1291

Emerich DF, Winn SR, Harper J, Hammang JP, Baetge EE, Kordower JH (1994) Implants of polymer-encapsulated human NGF-secreting cells in the non-human primate: rescue and sprouting of degenerating cholinergic basal forebrain neurons. J Comp Neurol 349:148–164

Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313–1317

Espinosa de los Monteros A, Zhao P, Huang C, Pan T, Chang R, Nazarian R, Espejo D, de Vellis J (1997) Transplantation of CG4 oligodendrocyte progenitor cells in the myelin-deficient rat brain results in myelination of axons and enhanced oligodendroglial markers. J Neurosci Res 50:872–887

Espinosa de los Monteros A, Baba H, Zhao PM, Pan T, Chang R, de Vellis J, Ikenaka K (2001) Remyelination of the adult demyelinated mouse brain by grafted oligodendrocyte progenitors. Neurochem Res 26:673–682

Farris W, Mansourian S, Chang Y, Lindsley L, Eckman EA, Frosch MP, Eckman CB, Tanzi RE, Selkoe DJ, Guenette S (2003) Insulin-degrading enzyme regulates the levels of insulin, amyloid beta protein and the beta-amyloid precursor protein intracellular domain in vivo. Proc Natl Acad Sci USA 100:4162–4167

Fischer W, Wictorin K, Björklund A, Williams LR, Varon S, Gage FH (1987) Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. Nature 329:65–68

Fisher LJ, Jinnah HA, Kale LC, Higgins GA, Gage FH (1991) Survival and function of intrastriatally grafted primary fibroblasts genetically modified to produce L-DOPA. Neuron 6:371–380

Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billinghurst LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU, Snyder EY (1998) Engraftable human neural stem cells respond to developmental cues, replace neurons and express foreign genes. Nat Biotechnol 16:1033–1039

Franklin RJ, Blakemore WF (1997) Transplanting oligodendrocyte progenitors into the adult CNS. J Anat 190:23–33

Freeman TB, Cicchetti F, Hauser RA, Deacon TW, Li XJ, Hersch SM, Nauert GM, Sanberg PR, Kordower JH, Saporta S, Isacson O (2000) Transplanted fetal striatum in Huntington's disease: phenotypic development and lack of pathology. Proc Natl Acad Sci USA 97:13877–13882

Gage FH (2000) Mammalian neural stem cells. Science 287:1433–1438

Garbuzova-Davis S, Willing AE, Milliken M, Saporta S, Zigova T, Cahill DW, Sanberg PR (2002) Positive effect of transplantation of hNT neurons (NTera 2/D1 cell-line) in a model of familial amyotrophic lateral sclerosis. Exp Neurol 174:169–180

Glaser T, Perez-Bouza A, Klein K, Brustle O (2005) Generation of purified Oligodendrocyte progenitors from embryonic stem cells. FASEB J 19:112–114

Goldman S (2005) Stem and progenitor cell-based therapy of the human central nervous system. Nat Biotechnol 7:862–871

Gottlieb DI (2002) Large scale sources of neural stem cells. Annu Rev Neurosci 25:381–407

Greenamyre JT, Shoulson I (1994) Huntington disease. In: Calne D (ed) Neurodegenrative disease. WB Saunders, Philadelphia, pp 65–704

Gumpel M, Lachapelle F, Gansmuller A, Baulac M, Baron van Evercooren A, Baumann N (1987) Transplantation of human embryonic oligodendrocytes into shiverer brain. Ann N Y Acad Sci 495:71–85

Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng HX et al (1994) Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. Science 264:1772–1775

Guzman R, De Los AA, Cheshier S, Choi R, Hoang S, Liauw J, Schaar B, Steinberg G (2008) Intracarotid injection of fluorescence activated cell-sorted CD49d-positive neural stem cells improves targeted cell delivery and behavior after stroke in a mouse stroke model. Stroke 39:1300–1306

Hagell P, Brundin P (2002) Cell survival and clinical outcome following intrastriatal transplantation in Parkinson disease. J Neuropath Exp Neurol 60:741–752

Hagell P, Schrag A, Piccini P, Jahanshahi M, Brown R, Rehncrona S, Widner H, Brundin P, Rothwell JC, Odin P, Wenning GK, Morrish P, Gustavii B, Björklund A, Brooks DJ, Marsden CD, Quinn NP, Lindvall O (1999) Sequential bilateral transplantation in Parkinson's disease: effects of the second graft. Brain 122:1121–1132

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353–356

Harper PS (1996) Huntington's disease. W.B Saunders, Philadelphia

Harper JM, Krishnan C, Darman JS (2004) Axonal growth of embryonic stem cell-derived motoneurons in vitro and in motoneuron-injured adult rats. Proc Natl Acad Sci USA 101:7123–7128

Harris GJ, Codori AM, Lewis RF, Schmidt E, Bedi A, Brandt J (1999) Reduced basal ganglia blood flow and volume in pre-symptomatic, gene-tested persons at-risk for Huntington's disease. Brain 122:1667–1678

Hefti F (1986) NGF promotes survival of septal cholinergic neurons after fimbrial transection. J Neurosci 6:2155–2161

Hemming ML, Patterson M, Reske-Nielsen C, Lin L, Isacson O, Selkoe DJ (2007) Reducing amyloid plaque burden via ex vivo gene delivery of an Aβ-degrading protease: a novel therapeutic approach to Alzheimer disease. PLoS Med 4(e262):1405–1416

Honmou O, Houkin K, Matsunaga T, Niitsu Y, Ishiai S, Onodera R, Waxman SG, Kocsis JD (2011) Intravenous administration of auto serum-expanded autologous mesenchymal stem cells in stroke. Brain 134:1790–1807

Hudson AJ (1990) Amyotrophic lateral sclerosis: concepts in pathogenesis and etiology. University of Toronto Press, Toronto

Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72:971–983

Hwang DH, Kim BG, Kim EJ, Lee SI, Joo IS, Suh-Kim H, Sohn S, Kim SU (2009a) Transplantation of human neural stem cells transduced with Olig2 transcription factor improves locomotor recovery and enhances myelination in the white matter of rat spinal cord following contusive injury. BMC Neurosci 10:117

Hwang DH, Lee HJ, Seok JI, Kim BG, Joo IS, Kim SU (2009b) Intrathecal transplantation of human neural stem cells over-expressing VEGF provide behavioral improvement, disease onset delay and survival extension in transgenic ALS mice. Gene Ther 16:1234–1244

Iwata N, Tsubuki S, Takaki Y, Shirotani K, Lu B, Gerard NP, Gerard C, Hama E, Lee HJ, Saido TC (2001) Metabolic regulation of brain abeta by neprilysin. Science 292:1550–1562

Jeong SW, Chu K, Kim MH, Kim SU, Roh JK (2003) Human neural stem cell transplantation in experimental intracerebral hemorrhage. Stroke 34:2258–2263

Jiao S, Gurevich V, Wolff JA (1993) Long-term correction of rat model of Parkinson's disease by gene therapy. Nature 362:450–453

Jin K, Mao X, Xie L, Galvan V, Lai B, Wang Y, Gorostiza O, Wang X, Greenberg DA (2010) Transplantation of human neural precursor cells in Matrigel scaffolding improves outcome from focal cerebral ischemia after delayed postischemic treatment in rats. J Cereb Blood Flow Metab 30(534–544):2010

Kang UJ, Fisher LJ, Joh TH, O'Malley KL, Gage FH (1993) Regulation of dopamine production by genetically modified primary fibroblasts. J Neurosci 13:5203–5211

Kawai H, Yamashita T, Ohta Y, Deguchi K, Nagotani S, Zhang X, Ikeda Y, Matsuura T, Abe K (2010) Tridermal tumorigenesis of induced pluripotent stem cells transplanted in ischemic brain. J Cereb Blood Flow Metab 30:1487–1493

Kawasaki H, Mizuseki K, Nishikawa S, Kaneko S, Kuwana Y, Nakanishi S, Nishikawa SI, Sasai Y (2000) Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron 28:31–40

Keene CD, Chang RC, Leverentz JB, Kopyov O, Perlman S, Hevner RF, Born DE, Bird TD, Montine TJ (2009) A patient with Huntington's disease and long-surviving fetal neural transplants that developed mass lesions. Acta Neuropath 117:329–338

Kerr DA, Lladó J, Shamblott MJ, Maragakis NJ, Irani DN, Crawford TO, Krishnan C, Dike S, Gearhart JD, Rothstein JD (2003) Human embryonic germ cell derivatives facilitate motor recovery of rats with diffuse motor neuron injury. J Neurosci 23:5131–5140

Kim SU (2004) Human neural stem cells genetically modified for brain repair in neurological disorders. Neuropathology 24:159–174

Kim JH, Auerbach JM, Rodríguez-Gómez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sánchez-Pernaute R, Bankiewicz K, McKay R (2002) Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 418:50–56

Kim TE, Lee HS, Lee YB, Hong SH, Lee YS, Ichinose H, Kim SU, Lee MA (2003) Sonic hedgehog and FGF8 collaborate to induce dopaminergic phenotype in Nurr-1 over-expressing neural stem cells. Biochem Biophys Res Comm 305:1040–1048

Kim SU, Park IH, Kim TH, Kim KS, Choi HB, Hong SH, Bang JH, Lee MA, Joo IS, Lee CS, Kim YS (2006) Brain transplantation of human neural stem cells transduced with tyrosine hydroxylase and GTP cyclohydrolase 1 provides functional improvement in animal models of Parkinson disease. Neuropathology 26:129–140

Kim M, Lee ST, Chu K, Kim SU (2008a) Stem cell-based cell therapy for Huntington disease: a review. Neuropathology 28:1–9

Kim SS, Yoo SW, Park TS, Ahn SC, Jeong HS, Kim JW, Chang DY, Cho KG, Kim SU, Huh Y, Lee JE, Lee SY, Lee YD, Suh-Kim H (2008b) Neural induction with neurogenin1 increases the therapeutic effects of mesenchymal stem cells in the ischemic brain. Stem Cells 26:2217–2228

Kim SU, Nagai A, Nakagawa E, Choi HB, Bang JH, Lee HJ, Lee MA, Lee YB, Park IH (2008c) Production and characterization of immortal human neural stem cell line with multipotent differentiation property. Methods Mol Biol 438:103–121

Kim SU, de Vellis J (2009) Stem cell-based cell therapy in neurological diseases: a review. J Neurosci Res 87:2183–2200

Kim KS, Lee HJ, Seo Y, Lim IJ, Kim SU (2011) Generation of human motoneurons from neural stem cells and motoneuron-base cell therapy in ALS mouse. Abstr Soc Neurosci 2011:558.06.

Kirks S, Shim JW, Piao J, Ganat YM, Wakeman DR, Xie Z, Carrillo-Reid L, Auyeung G, Antonacci C, Buch A, Yang L, Beal MF, Surmeier DJ, Kordower JH, Tabar V, Studer L (2011) Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. Nature 480:547–551

Kish SJ, Shannak K, Hornykiewitcz O (1988) Uneven pattern of dopamine loss in the striatum of patients with idiopathic Parkinson's disease. Pathophysiologic and clinical implications. N Eng J Med 318:876–880

Klein SM, Behrstock S, McHugh J, Hoffmann K, Wallace K, Suzuki M, Aebischer P, Svendsen CN (2005) GDNF delivery using human neural progenitor cells in a rat model of ALS. Human Gene Ther 16:509–521

Kondziolka D, Wechsler L, Goldstein S, Meltzer C, Thulborn KR, Gebel J, Jannetta P, DeCesare S, Elder EM, McGrogan M, Reitman MA, Bynum L (2000) Transplantation of cultured human neuronal cells for patients with stroke. Neurology 55:565–569

Kordower JH, Tuszinski MH (1999) CNS regeneration: basic sciences and clinical advances. Academic, San Diego

Kordower JH, Goetz CG, Freeman TB, Olanow CW (1997a) Doparminergic transplants in patients with Parkinson's disease: neuroanatomical correlates of clinical recovery. Exp Neurol 144:41–46

Kordower JH, Chen EY, Winkler C, Fricker R, Charles V, Messing A, Mufson EJ, Wong SC, Rosenstein JM, Björklund A, Emerich DF, Hammang J, Carpenter MK (1997b) Grafts of EGFresponsive neural stem cells derived from GFAP-hNGF transgenic mice: tropic and tropic effects in a rodent model of Huntington's disease. J Comp Neurol 387:96–113

Kurozumi K, Nakamura K, Tamika T, Kawano Y, Koruna M, Hirai S, Uchida H, Sasaki K, Ito Y, Kato K, Honmou O, Houkin K, Date I, Hamada H (2004) BDNF gene-modified mesenchymal stem cells promote functional recovery and reduce infarct size in the rat middle cerebral artery occlusion model. Mol Ther 9:189–197

Lachapelle F, Gumpel M, Baulac C, Jacque C (1983) Transplantation of fragments of CNS into the brain of shiverer mutant mice: extensive myelination of transplanted oligodendrocytes. Dev Neurosci 6:326–334

Lang AE, Lozano AM (1998a) Parkinson's disease. First of two parts. N Engl J Med 339:1044–1053

Lang AE, Lozano AM (1998b) Parkinson's disease. Second of two parts. N Engl J Med 339:1130–1143

Le Gros Clark WE (1940) Neuronal differentiation in implanted foetal cortical tissue. J Neurol Psychiatry 3:263–284

Learish RD, Brustle O, Zhang SC, Duncan ID (1999) Intraventricular transplantation of oligodendrocyte progenitors into a fetal myelin mutants in widespread formation of myelin. Ann Neurol 46:716–722

Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol 18:675–679

Lee ST, Chu K, Park JE, Lee K, Kang L, Kim SU, Kim M (2005) Intravenous administration of human neural stem cells induces functional recovery in Huntington's disease rat model. Neurosci Res 52:243–249

Lee ST, Park JE, Lee K, Kang L, Chu K, Kim SU, Kim M, Roh JK (2006) Noninvasive method of immortalized neural stem-like cell transplantation in an experimental model of Huntington's disease. J Neurosci Meth 52:250–254

Lee HJ, Kim KS, Kim EJ, Choi HB, Lee KH, Park IH, Ko Y, Jeong SW, Kim SU (2007a) Brain transplantation of human neural stem cells promotes functional recovery in mouse intracerebral hemorrhage stroke model. Stem Cells 25:211–224

Lee HJ, Kim KS, Kim EJ, Park IH, Kim SU (2007b) Human neural stem cells over-expressing VEGF provide neuroprotection, angiogenesis and functional recovery in mouse stroke model. PLoS One 1:e156

Lee ST, Chu K, Jung KH, Kim SJ, Kim DH, Kang KM, Hong NH, Kim JH, Ban JJ, Park HK, Kim SU, Park CG, Lee SK, Kim M, Roh JK (2008) Anti-inflammatory mechanism of intravascular neural stem cell transplantation in hemorrhagic stroke. Brain 131:616–629

Lee HJ, Park IH, Kim HJ, Kim SU (2009a) Human neural stem cells overexpressing glial cell line derived neurotrophic factor (GDNF) promote functional recovery and neuroprotection in experimental cerebral hemorrhage. Gene Ther 16:1066–1076

Lee HJ, Kim MK, Kim HJ, Kim SU (2009b) Human neural stem cells genetically modified to overexpress Akt1 provide neuroprotection and functional improvement in mouse stroke model. PLoS One 4:e5586

Lee HJ, Lim IJ, Lee MC, Kim SU (2010a) Human neural stem cells genetically modified to overexpress BDNF promote functional recovery and neuroprotection in mouse stroke model. J Neurosci Res 88:3282–3294

Lee JS, Hong JM, Moon GJ, Lee PH, Ahn YH, Bang OY (2010b) A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. Stem Cells 28:1099–1106

Lee HJ, Lim IJ, Park SW, Kim YB, Kim SU (2012) Human neural stem cells genetically modified to express human nerve growth factor gene restore cognition in ibotenic acid-induced cognitive dysfunction. Cell Transplant 2012 Apr 20 [Epub ahead of print]

Li P, Tessler A, Han SS, Fischer I, Rao MS, Selzer ME (2005) Fate of immortalized human neuronal progenitor cells transplanted in rat spinal cord. Arch Neurol 62:223–229

Lindvall O, Kokaia Z (2006) Stem cells for the treatment of neurological disorders. Nature 441:1094–1096

Lindvall O, Brundin P, Widner H, Rehncrona S, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, Marsden CD (1990) Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. Science 247:574–577

Lindvall O, Kokaia Z, Martinez-Serrano A (2004) Stem cell therapy for human neurodegenerative disorders-how to make it work. Nat Med 10(suppl):S42–S50

Liu S, Qu Y, Stewart TJ, Howard MJ, Chakrabortty S, Holekamp TF, McDonald JW (2000) Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after transplantation. Proc Natl Acad Sci USA 97:6126–6131

Lu QR, Yuk D, Alberta JA, Zhu Z, Pawlitzky I, Chan J, McMahon AP, Stiles CD, Rowitch DH (2000) Sonic hedgehog-regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. Neuron 25:317–329

Lyons MK (2011) Deep brain stimulation: current and future clinical applications. Mayo Clin Proc 86:662–672
Marr RA, Rockenstein E, Mukherjee A, Kindy MS, Hersh LB, Gage FH, Verma IM, Masliah E (2003) Neprilysin gene transfer reduces human amyloid pathology in transgenic mice. J Neurosci 23:1992–1996
Marshall J, Thomas DJ (1988) Cerebrovascular disease. In: Asbury A, McKhann G, McDonald W (eds) Diseases of the nervous system. WB Saunders, Philadelphia, pp 1101–1135
McBride JL, Behrstock SP, Chen EY, Jakel RJ, Siegel I, Svendsen CN, Kordower JH (2004) Human neural stem cell transplants improve motor function in a rat model of Huntington's disease. J Comp Neurol 475:211–219
McFarlin DE, McFarland HF (1982) Multiple sclerosis. N Eng J Med 307:1183–1188
McKay RG (1997) Stem cells in the central nervous system. Science 276:66–71
Melchor JP, Pawlak R, Strickland S (2003) The tissue plasminogen activator-plasminogen proteolytic cascade accelerates amyloid-beta degradation and inhibit Abeta-induced neurodegeneration. J Neurosci 23:8867–8871
Miles GB, Yohn DC, Wichterle H (2004) Functional properties of motoneurons derived from mouse embryonic stem cells. J Neurosci 24:7848–7858
Miller BC, Eckman EA, Sambamurti K, Dobbs N, Chow KM, Eckman CB, Hersh LB, Thiele DL (2003) Amyloid-beta peptide levels in brain are inversely correlated with insulysin activity levels in vivo. Proc Natl Acad Sci USA 100:6221–6226
Miltrecic D, Nicaise C, Gajovic S, Pochet R (2010) Distribution, differentiation and survival of intravenously administered neural stem cells in a rat model of amuyotrophic lateral sclerosis. Cell Transplant 19:537–548
Modo M, Stroemer RP, Tang E, Patel S, Hodges H (2002) Effects of implantation site of stem cell grafts on behavioral recovery from stroke damage. Stroke 33:2270–2278
Moghadam FH, Alaie H, Karbalaie K, Tanhaei S, Nasr Esfahani MH, Baharvand H (2009) Transplantation of primed or unprimed mouse embryonic stem cells derived neural precursor cells improve cognitive function in Alzheimerian rats. Differentiation 78:59–68
Mueller-Steiner S, Zhou Y, Arai H, Roberson ED, Sun B, Chen J, Wang X, Yu G, Esposito L, Mucke L, Gan L (2006) Antiamyloidogenic and neuroprotective functions of cathepsin B: implications for Alzhemer's disease. Neuron 51:703–714
Musiał A, Bajda M, Malawska B (2007) Recent developments in cholinesterase inhibitors for Alzheimer's disease treatment. Curr Med Chem 14:2654–2679
Nagai A, Kim WK, Lee HJ, Jeong HS, Kim KS, Hong SH, Park IH, Kim SU (2007) Multilineage potential of stable human mesenchymal stem cell line derived from fetal marrow. PLoS One 2:e1272
Nakao N, Itakura T (2000) Fetal tissue transplants in animal models of Huntington's disease: The effects on damaged neuronal circuitry and behavioral deficits. Prog Neurobiol 61:313–338
Nistor GI, Totoiu MO, Haque N, Carpenter MK, Keirstead HS (2005) Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. Glia 49:385–396
Olanow CW, Kordower J, Freeman T (1996) Fetal nigral transplantation as a therapy for Parkinson's disease. Trends Neurosci 19:102–109
Park IH, Zhao R, West JA (2008) Reprogramming of human somatic cells to pluripotency with defined factors. Nature 415:141–146
Park D, Joo SS, Kim TK, Choi YJ, Lee SH, Kang H, Lee HJ, Kim YB, Kim SU (2012a) Human neural stem cells over expressing choline acetyltransferase gene recover cognitive function of kainic acid-Induced learning and memory deficit animals. Cell Transplant 21:365–371
Park D, Lee HJ, Joo SS, Matsuo A, Tooyama I, Kim YB, Kim SU (2012b) Human neural stem cells over-expressing choline acetyltransferase restore cognition in rat model of cognitive dysfunction. Exp Neurol 234:521–526
Paty D, Ebers GC (1998) Multiple sclerosis. FA Davis, Philadelphia
Pendharkar AV, Chua JY, Andres RH, Wang N, Gaeta X, Wang H, De A, Choi R, Chen S, Rutt BK, Gambhir SS, Guzman R (2010) Biodistribution of neural stem cells after intravascular therapy for hypoxic-ischemia. Stroke 41:2064–2070

Pérez-Navarro E, Canudas AM, Akerund P, Alberch J, Arenas E (2000) Brain-derived neurotrophic factor, NT-3 and NT-3/4 prevent the death of striatal projection neurons in rodent model of Huntington's disease. J Neurochem 75:2190–2199

Perlow MJ, Freed WJ, Hoffer BJ, Seiger A, Olson L, Wyatt RJ (1979) Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. Science 204:643–647

Pluchino S, Zanotti L, Rossi B, Brambilla E, Ottoboni L, Salani G, Martinello M, Cattalini A, Bergami A, Furlan R, Comi G, Constantin G, Martino G (2005) Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. Nature 436:266–271

Ramon y Cajal S (1928) Degeneration and regeneration of the nervous system. Hafner, New York

Ransom SW (1909) Transplantation of spinal ganglion into the brain. Q Bull Northwest Univ Med Sch 11:176–178

Redmond DE Jr, Bjugstad KB, Teng YD, Ourednik V, Ourednik J, Wakeman DR, Parsons XH, Gonzalez R, Blanchard BC, Kim SU, Gu Z, Lipton SA, Markakis EA, Roth RH, Elsworth JD, Sladek JR Jr, Sidman RL, Snyder EY (2007) Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. Proc Natl Acad Sci USA 104:12175–12180

Renfranz PJ, Cunningham M, McKay R (1991) Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. Cell 66:713–729

Roberts TJ, Price J, Williams SC, Modo M (2006) Preservation of striatal tissue and behavioral function after neural stem cell transplantation in a rat model of Huntington's disease. Neuroscience 139:1187–1199

Rowland LP, Shneider NA (2001) Amyotrophic lateral sclerosis. New Eng J Med 344:1688–1700

Ryu JK, Kim J, Cho SJ, Hatori K, Nagai A, Choi HB, Lee MC, McLarnon JG, Kim SU (2004) Proactive transplantation of human neural stem cells blocks neuronal cell death in rat model of Huntington disease. Neurobiol Disease 16:68–77

Ryu MY, Lee MA, Ahn YH, Kim KS, Yoon SH, Snyder EY, Cho KG, Kim SU (2005) Brain transplantation of genetically modified neural stem cells in parkinsonian rat. Cell Transplant 14:193–202

Sah DW, Ray J, Gage F (1997) Bipotent progenitor cell lines from the human CNS. Nat Biotechnol 15:574–580

Saporta S, Borlongan CV, Sanberg PR (1999) Neural transplantation of human teratocarcinoma neurons into ischemic rats. A quantitative dose-response analysis of cell survival and behavioral recovery. Neuroscience 180:519–525

Savitz SI, Rosenbaum DM, Dinsmore JH, Wechsler LR, Caplan LR (2002) Cell transplantation for stroke. Ann Neurol 52:266–275

Seilhean D, Gansmüller A, Baron-Van Evercooren A, Gumpel M, Lachapelle F (1996) Myelination by transplanted human and mouse CNS tissue after long-term cryopreservation. Acta Neuropath 91:82–88

Seminatore C, Polentes J, Ellman D, Kozubenko N, Itier V, Tine S, Tritschler L, Brenot M, Guidou E, Blondeau J, Lhuillier M, Bugi A, Aubry L, Jendelova P, Sykova E, Perrier AL, Finsen B, Onteniente B (2010) The postischemic environment differentially impacts teratoma or tumor formation after transplantation of human embryonic stem cell-derived neural progenitors. Stroke 41:153–159

Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, Blumenthal PD, Huggins GR, Gearhart JD (1998) Derivation of pluripotent stem cells from cultured juman primordial germ cells. Proc Natl Acad Sci USA 95:13726–13731

Shim JW, Koh HC, Chang MY, Roh E, Choi CY, Oh YJ, Son H, Lee YS, Studer L, Lee SH (2004) Enhanced in vitro midbrain dopamine neuron differentiation, dopaminergic function, neurite outgrowth, and 1-methyl-4-phenylpyridium resistance in mouse embryonic stem cells overexpressing Bcl-XL. J Neurosci 24:843–852

Sinden JD, Rashid-Doubell F, Kershaw TR, Nelson A, Chadwick A, Jat PS, Noble MD, Hodges H, Gray JA (1997) Recovery of spatial learning by grafts of a conditionally immortalized hippocampal neuroepithelial cell line into the ischemia-lesioned hippocampus. Neuroscience 23:599–608

Snyder EY, Deitcher DL, Walsh C (1992) Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. Cell 68:33–51

Song J, Lee ST, Kang W, Park JE, Chu K, Lee SE, Hwang T, Chung H, Kim M (2007) Human embryonic stem cell-derived neural precursor transplantation induced rotational behavior in rats with unilateral quinolinic acid lesions. Neurosci Lett 423:58–61

Storkebaum E, Lambrechts D, Dewerchin M, Moreno-Murciano MP, Appelmans S, Oh H, Van Damme P, Rutten B, Man WY, De Mol M, Wyns S, Manka D, Vermeulen K, Van Den Bosch L, Mertens N, Schmitz C, Robberecht W, Conway EM, Collen D, Moons L, Carmeliet P (2005) Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS. Nat Neurosci 8:85–92

Takagi Y, Takahashi J, Saiki H, Morizane A, Hayashi T, Kishi Y, Fukuda H, Okamoto Y, Koyanagi M, Ideguchi M, Hayashi H, Imazato T, Kawasaki H, Suemori H, Omachi S, Iida H, Itoh N, Nakatsuji N, Sasai Y, Hashimoto N (2005) Dopaminergic neurons generated from monkey ES cells function in a Parkinson primate model. J Clin Invest 115:102–108

Takahashi K, Tanabe K, Ohnuki M (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872

Takebayashi H, Yoshida S, Sugimori M, Kosako H, Kominami R, Nakafuku M, Nabeshima Y (2000) Dynamic expression of bHLH olig family members: implication of Olig2 in neuron and OLigodendrocyte differentiation and identification of a new member, Olig3. Mech Devel 99:143–148

Temple S (2001) The development of neural stem cells. Nature 414:112–117

Terry AV, Buccafusco JJ (2003) The cholinergic hypothesis of age and Alzheimer's diseaserelated cognitive deficits: recent challenges and their implications for novel drug development. J Pharmacol Exp Ther 306:821–827

Thieben MJ, Duggins AJ, Good CD, Gomes L, Mahant N, Richards F, McCusker E, Frackowiak RS (2002) The distribution of structural neuropathology in pre-clinical Huntington's disease. Brain 125:1815–1828

Thompson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell line derived from human blastocysts. Science 282:1145–1147

Thompson WG (1890) Successful brain grafting. N Y Med J 51:701–702

Tuszynski DW (2002) Gene therapy for neurodegenerative disorders. Lancet Neurol 1:51–57

Tuszynski DW, U HS, Amaral DG, Gage FH (1990) Nerve growth factor infusion in primate brain reduces lesion-induced cholinergicneuronal degeneration. J Neurosci 10:3604–3614

Tuszynski MH, Thal L, Pay M, Salmon DP, U HS, Bakay R, Patel P, Blesch A, Vahlsing HL, Ho G, Tong G, Potkin SG, Fallon J, Hansen L, Mufson EJ, Kordower JH, Gall C, Conner J (2005) A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. Nat Med 11:551–555

Vasey EM, Dottori M, Jamshidi P (2010) Comparison of transplant efficacy between spontaneucely derived and noggin-primed human embryonic stem cell neural precursors in the quinOLinic acid rat model of Huntington's disease. Cell Transplant 19:1055–1062

Veizovic T, Beech JS, Stroemer RP, Watson WP, Hodges H (2001) Resolution of stroke deficits following contalateral grafts of conditionally immortlized neuroepithelial stem cells. Stroke 32:1012–1019

Visnyei K, Tatsukawa KJ, Erickson RI, Simonian S, Oknaian N, Carmichael ST, Kornblum HI (2006) Neural progenitor implantation restores metabolic deficits in the brain following striatal quinolinic acid lesion. Exp Neurol 197:465–474

Wagner J, Akerud P, Castro DS, Holm PC, Canals JM, Snyder EY, Perlmann T, Arenas E (1999) Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes. Nat Biotechnol 17:653–659

Wang Q, Matsumoto Y, Shindo T, Miyake K, Shindo A, Kawanishi M, Kawai N, Tamika T, Nagao S (2006) Neural stem cells transplantation in cortex in a mouse model of Alzheimer's disease. J Med Invest 53:61–69

Watabe K, Ohashi T, Sakamoto T, Kawazoe Y, Takeshima T, Oyanagi K, Inoue K, Eto Y, Kim SU (2000) Rescue of lesioned adult rat spinal motoneurons by adenoviral gene transfer of glial cell line-derived neurotrophic factor. J Neurosci Res 60:511–519

Werning M, Zhao J, Pruszak J, Hedlund E, Fu D, Soldner F, Broccoli V, Constantine-Paton M, Isacson O, Jaenisch R (2008) Neurons derived from reprogramed fibroblasts functionally

integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. Proc Natl Acad Sci USA 105:5856–5861

Whitehouse PJ, Price DL, Clark AW, Coyle JT, DeLong MR (1981) Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. Ann Neurol 10:122–126

Wichterle H, Lieberam I, Porter JA, Jessell TM (2002) Directed differentiation of embryo stem cells into motor neurons. Cell 110:385–397

Windrem MS, Nunes MC, Rashbaum WK, Schwartz TH, Goodman RA, McKhann G, Roy NS, Goldman SA (2004) Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain. Nat Med 10:93–97

Wolff JA, Fisher LJ, Xu L, Jinnah HA, Langlais PJ, Iuvone PM, O'Malley KL, Rosenberg MB, Shimohama S, Friedmann T et al (1989) Grafting fibroblasts genetically modified to produce L-dopa in a rat model of Parkinson disease. Proc Natl Acad Sci USA 86:9011–9014

Wu S, Sasaki A, Yoshimoto R, Kawahara Y, Manabe T, Kataoka K, Asashima M, Yuge L (2008) Neural stem cells improve learning and memory in rats with Alzheimer's disease. Pathobiology 75:186–194

Xu L, Yan J, Chen D, Welsh AM, Hazel T, Johe K, Hatfield G, Koliatsos VE (2006) Human neural stem cell grafts ameliorate motor neuron disease in SOD1 transgenic rats. Transplantation 82:865–875

Yamazaki N, Kato K, Kurihara E, Nagaoka A (1991) Cholinergic drugs reverse AF64A-induced impairment of passive avoidance learning in rats. Psychopharmacology (Berl) 103:215–222

Yandava B, Billinghurst L, Snyder E (1999) Global cell replacement is feasible via neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain. Proc Natl Acad SciUSA 96:7029–7034

Yasuhara T, Matsukawa N, Hara K, Yu G, Xu L, Maki M, Kim SU, Borlongan CV (2006) Transplantation of neural stem cells exerts neuroprotection in a rat model of Parkinson disease. J Neurosci 26:124497–124511

Yu J, Vodyanik MA, Smuga-Otto K (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920

Zhang SC, Ge B, Duncan ID (1999) Adult brain retains the potential to generate Oligodendroglial progenitors with extensive myelination capacity. Proc Natl Acad Sci USA 96:4089–4094

Zhao LR, Duan WM, Reyes M, Keene CD, Verfaillie CM, Low WC (2002) Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischmica brain of rats. Exp Neurol 174:11–20

Zheng C, Nennesmo I, Fadeel B, Henter JI (2004) Vascular endothelial growth factor prolongs survival in a transgenic mouse model of ALS. Ann Neurol 56:564–567

Zhou Q, Wang S, Anderson DJ (2000) Identification of a novel family of oligodendrocyte lineage specific basic helix loop helix transcription factors. Neuron 25:331–343

Chapter 28
Regenerative Therapy for Central Nervous System Trauma

Kewal K. Jain

Abstract Functional regeneration and not merely structural restoration is important in the central nervous system (CNS) following loss of tissue due to trauma. Spontaneous regeneration in the CNS is poor due to a number of reasons, mainly the presence of inhibitory factors. This chapter reviews some of the mechanism of this inhibition on which the strategies to promote regeneration in the CNS are based. These strategies are considered for application in traumatic brain injury (TBI) and spinal cord injury (SCI) separately. Degradation of inhibitors such as chondroitin sulfate proteoglycans in the glial scar at the site of SCI by application of chondroitinase ABC promotes regeneration of corticospinal tract axons in experimental animals. Inhibitors of axonal regeneration in myelin include Nogo, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein. These can be blocked with antibodies or peptides to facilitate regeneration after SCI. Apart from acute TBI, chronic traumatic encephalopathy is being increasingly recognized as a cause of cognitive impairment and strategies for regeneration are similar to those for neurodegenerative disorders. Cell and gene therapies are under investigation for CNS regeneration. Developments in nanobiotechnology also show potential for CNS repair. However, experimental work in CNS regeneration has not yet been translated into clinical use. Combination of approaches, including stem cell transplantation with nanoscaffolds, supplemented with pharmacological enhancement of regeneration, hyperbaric oxygen, and physical therapies are promising for functional regeneration of the CNS following trauma.

K.K. Jain, M.D., FRACS, FFPM (✉)
Jain PharmaBiotech, Blaesiring 7, 4057 Basel, Switzerland
e-mail: jain@pharmabiotech.ch

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_28, © Springer Science+Business Media Dordrecht 2013

28.1 Introduction

The term "regeneration" is used to describe the sum total of activities leading to re-growth of cells and tissues of the body. It includes both anatomical and physiological structures; however, structural regeneration does not necessarily lead to restoration of function. The term "functional regeneration" implies recovery of the function that can occur without regeneration by compensatory mechanisms. Functional regeneration is important for recovery of the central nervous system (CNS) consisting of the brain and the spinal cord, following damage or loss of cells and tissues resulting from traumatic brain injury (TBI) and spinal cord injury (SCI).

28.2 Historical Background

Although regeneration was known to occurs to a variable degree in most body tissues, neural tissues (excepting peripheral nerves) were considered to be non-regenerative, an idea that was recognized as early as 1550 BC and well documented during the nineteenth century (Mitchell 1872). In the earlier part of the twentieth century, Ramon y Cajal reached the following conclusion in his monumental work on degeneration and regeneration of the nervous system: Once the development has ended, the founts of growth and regeneration of the axons and dendrites dry up irrevocably. In adult centers the nerve paths are something fixed, ended, immutable (Ramon y Cajal 1959). The view of axonal regeneration in the CNS as abortive or poor remained widely accepted for several decades. Evidence started to emerge during the last quarter of the twentieth century that, under certain circumstances, regeneration could occur successfully in the mammalian CNS. Discoveries in neurobiology have provided an insight into possible ways in which regeneration in the CNS may be encouraged.

Hughling Jackson, in his book "Principal of Compensation", explained the functional recovery that occurs following damage to the CNS, which was based on his theory of cerebral localization (York and Steinberg 1994). Functional recovery is related to plasticity of the CNS, i.e. its ability to adapt its structural organization, both anatomically and functionally, to new situations emerging during its maturation, in addition to those resulting from injuries. The concept of neuroplasticity was developed during the early part of the twentieth century (Goldstein 1931). Investigators in this field had already recognized the plasticity exhibited by brain microglia during development and under pathological conditions (del Rio-Hortega 1932). A further advance is the concept of reactive synaptogenesis, whereby the neighboring neurons make new synaptic contacts to replace those lost and play a major role in the restoration of function following brain damage (Cotman and Scheff 1979).

Some of the basic concepts of regeneration and repair taking place after CNS injury have led to strategies for treatment and rehabilitation of patients with brain damage. Initial attempts to use neural grafts to repair the damage in experimental animals took place more than a century ago (Thompson 1890). During the last quarter of the twentieth century, neural grafting techniques were refined and investigated for TBI and SCI.

28.3 Basics of CNS Regeneration

Primary sensory neurons with cell bodies in the dorsal root ganglia have two branches: (1) a peripheral axon that regenerates itself if injured; and (2) a central axon that enters the CNS and does not regenerate after injury. The local environment of these branches explains the difference in regeneration: The peripheral axon contains Schwann cells, whereas the central axon contains oligodendrocytes and astrocytes. In the peripheral nervous system, myelin debris is cleared promptly, and Schwann cells dedifferentiate and down-regulate expression of the myelin protein, thus facilitating regeneration. The composition and organization of extracellular matrix in CNS lesions is a hindrance to regeneration. However, regeneration of CNS axons has been demonstrated in vivo after implantation of peripheral nervous tissue. Furthermore, regeneration of dorsal column fibers has been demonstrated beyond the lesion site in adult spinal cord injury by a preconditioning peripheral nerve lesion 1–2 weeks before the spinal cord lesion (Neumann and Woolf 2000).

28.3.1 Factors that Influence Regeneration in the Central Nervous System

Intrinsic factors that influence regeneration in the CNS. Various intrinsic factors that modulate regeneration in the CNS are listed in Table 28.1 and described in the following text. Neurotrophic factors are the most important of all the factors influencing regeneration.

28.3.2 Causes of Lack of Regeneration in the CNS

The CNS regenerative process is unsuccessful for three reasons: (1) neurons are highly susceptible to death after CNS injury; (2) multiple inhibitory factors in the CNS environments hinder regeneration; and (3) the intrinsic growth capacity of postmitotic neurons is constitutively reduced. Research is providing an insight into these areas and will form the basis of strategies to promote regeneration of the CNS.

28.3.2.1 Factors Inhibiting Regeneration in the CNS

Glial scar. A glial scar containing extracellular matrix molecules including chondroitin sulfate proteoglycans develops at the site of injury and prevents regeneration.

Table 28.1 Factors that influence regeneration in the central nervous system

Factors	Role in regeneration and recovery
Neurotrophic factors	Cell survival, axon growth-cone stimulation, synapse regeneration
Neuroprotective gene expression	Expression of genes such as Bcl-2 and c-fos/jun may occur within minutes of an acute brain injury and are a determinant of eventual recovery
Neural stem cells	Neural stem cells can migrate to the site of injury in the brain and participate in regeneration
Cadherins	These are involved in synaptogenesis in the CNS
Intracellular levels of cyclic nucleotide in the neurons	These influence the capacity of mature CNS neurons to initiate and maintain a regrowth response
Innate immune system, represented by activated macrophages	This can facilitate the processes of regeneration in the severed spinal cord
Inducible nitric oxide synthase	This is not usually present in the brain but can be detected in the brain following injury and may be required for adequate repair
Activin	Strong expression of activin is seen in repair processes of the brain and may have a role in neuroprotection. Although a transient overexpression of activin after tissue injury might be beneficial for the repair process, sustained expression of activin could be detrimental to regeneration

Neurite outgrowth inhibitors. Various growth inhibitors are found in a glial scar. These include the following:

- Myelin-associated inhibitors of axonal regeneration.
- Astrocytes produce tenascin, brevican, and neurocan.
- Meningeal cells produce NG2 and other proteoglycans.
- Activated microglia produce free radicals, nitric oxide, and arachidonic acid derivatives.

Three inhibitors of axonal regeneration have been identified in myelin: Nogo, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein. Postmortem histopathological studies on SCI patients have shown that NG2 and phosphacan are both present in the evolving astroglial scar and, therefore, might have played an important role in the blockade of successful CNS regeneration (Buss et al. 2009). All of these proteins induce growth cone collapse and inhibit neurite outgrowth. Three of the four known myelin inhibitors, Nogo66, Myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp), although very different structurally, interact with the same receptor, NgR. They exert their inhibitory effects by binding the NgR receptor that transduces the inhibitory signal to the cell interior via transmembrane co-receptors LINGO-1 and p75(NTR) or TROY. Although the receptor(s) for amino-Nogo-A are unknown, amino-Nogo-A and NgR ligands mutually activate the small GTPase RhoA (Walmsley and Mir 2007). However, the absence of NgR alone has no effect on inhibition of neurite

outgrowth in culture, and a second receptor, PirB, was described for these myelin inhibitors (Filbin 2008).

In vitro neurite outgrowth studies have demonstrated that a significant part of myelin inhibition is mediated by Ephrin-B3, which remarkably equals the inhibitory activity of Nogo, MAG and OMgp combined. Loss of EphA4, a receptor, leads to improved axon regeneration and functional recovery. Several other guidance cues are expressed by oligodendrocytes. Sema4D, a transmembrane class 4 Semaphorin, is transiently upregulated in oligodendrocytes that surround the lesion site after adult CNS injury and may inhibit CNS axonal regeneration. Another member of the Semaphorin family, Sema5A, has also been shown to be expressed by oligodendrocytes; it induces growth cone collapse and inhibits neurite growth.

Humoral autoantibodies. Autoimmune responses directed against the CNS are generally considered pathogenic in nature, but autoreactive antibodies can also enhance endogenous myelin repair.

28.3.2.2 Role of Glial Cells in CNS Injury and Regeneration

Glial cells consist of microglia, which have a phagocytic function, and macroglia (astrocytes and oligodendrocytes). Astrocytes provide structural, trophic, and metabolic support to neurons and modulate synaptic activity. Therefore, impairment of astrocyte functions in TBI can compromise neuron survival. Functions of astrocytes that are known to influence neuronal survival include glutamate metabolism, free-radical scavenging, and the production of cytokines and nitric oxide. Neuron regeneration after TGI is influenced by the release of neurotrophic factors by astrocytes. Therapeutic approaches to TBI should be aimed at restoring the functions of both neurons and glial cells. Glial cells may also contribute to scar formation.

A study has shown how astroglia can be directly converted into the two main classes of cortical neurons, excitatory as well as inhibitory, by the selective transduction of transcription factors, specific proteins that regulate the transcription of DNA (Heinrich et al. 2010). This approach may provide new therapies for neurodegenerative diseases.

28.3.2.3 Role of Neurotrophic Factors in Neuronal Regeneration

The role of neurotrophic factors during neuronal regeneration differs little from their role during neuronal development in the expression of cytoskeletal genes or cellular protein synthesis, suggesting that regulatory events during regeneration recapitulate the patterns found during development.

Nerve growth factor. Within the central nervous system, the main neuronal system regulated by nerve growth factor is that of basal forebrain cholinergic neurons, which send topographically organized projections to the hippocampus and cerebral neocortex. Regeneration in the adult mammalian central nervous system has been viewed pessimistically in the past. Rapid progress of concepts and tools in developmental

biology has been applied to approach the questions of regeneration. Important aims are cell survival, re-initiation of axon growth, target finding, and formation of functional connections. Significant recent developments include the availability of recombinant neurotrophic factors and stem cells for repair of the nervous system.

Neurotrophic factors and synapse regeneration. Synapses are the final common pathway for information exchange in the nervous system. They mediate a wide range of activities from a simple reflex arc to learning and memory. Synapse formation plays an important role in neuronal regeneration and survival. The presynaptic and postsynaptic parts of the synapse are separated by a synaptic cleft. At the neuromuscular junction, a specialized extracellular matrix known as the synaptic basal lamina occupies this cleft. Neurotransmitters such as acetylcholine or glutamate are released from the presynaptic vesicles, traverse the cleft, and bind to their receptors on the postsynaptic membrane. The signal is terminated by the reuptake or enzymatic destruction of the neurotransmitters. Synapses are formed by the expression of specific gene products such as synaptic vesicle proteins and neurotransmitter receptors. This process is regulated to some extent by a combination of neurotrophic factors and electrical activity.

28.3.2.4 Cadherins

These are found in the synaptic cleft near the transmitter release zone and may provide a molecular basis for the adhesive interactions between opposing synaptic membranes. Thus, they play a role in the formation and maintenance of synapses. Cadherins might directly regulate cell signaling to modulate synaptic connectivity.

Pan-cadherin is a good biomarker for neuronal recovery after cortical injury. Immunohistochemical staining of the injured cortex for pan-cadherin revealed a significant increase in staining in experimental animals treated with topical application of NEP 1–4, an inhibitory peptide that neutralizes Nogo-A, and preserves neuronal structures (Atalay et al. 2008).

28.3.2.5 Various Factors that Influence Plasticity in the CNS

The adult cortex undergoes plastic changes that are dependent on neuronal activity. Plasticity in the CNS following injury is influenced by several factors:

- The brain possesses a certain degree of biological plasticity that diminishes with age.
- Plasticity can be limited by a progressive neurodegenerative disease or severe damage to the brain.
- There is less room for plasticity in spinal cord lesions than in the cerebral hemispheres.
- Secondary damage that results from traumatic lesions of the CNS may reduce the role of plasticity in recovery.
- Neurotrophins and their receptors play a role in this plasticity.

Table 28.2 A classification of approaches to regeneration of the CNS

Inhibiting the factors that impede regeneration in the CNS
Reducing or eliminating scar formation
Providing cues to axons for regeneration
Repair of the injured nerve fibers
Promoting growth of neural tissues to replace the loss
Use of stem cells
Use of neural as well as non-neural cells
Hyperbaric oxygen for neuroprotection and mobilization of intrinsic stem cells
Physical agents to accelerate growth of nerve cells
Pharmacological agents to promote growth of neural tissues
Restoration of neurotransmission
Struts for tissue engineering
Self degrading biomaterials
Nanomaterials

Rapid and opposing effects of brain-derived neurotrophic factor and nerve growth factor on the functional organization of the adult cortex in the rat indicate that neurotrophins can modulate stimulus-dependent activity in the adult cortex. Such studies also suggest a role for neurotrophins in regulating adult cortical plasticity. Current knowledge enables some manipulation of plasticity and the induction of functional changes beneficial for vision.

GAP-43 plays an important role in axonal plasticity by guiding growth cones rather than supporting axonal elongation. The molecule GAP-43 is key to initiating axon growth, whereas other genes are necessary to develop a full regenerative program. Addition of GAP-43 gene can induce the formation of branched plexuses typical of sprouting growth.

28.4 Approaches to Regeneration of the CNS

Several technologies are being used to facilitate regeneration and repair of the CNS. A classification of these technologies is shown in Table 28.2.

28.4.1 Inhibiting the Factors that Impede Regeneration in the CNS

Various factors that inhibit regeneration following injury to the CNS have been identified in the earlier sections. In order to overcome the inhibitory environment of the glial scar, treatments should enhance the ability of neurons to elongate and manipulate the extrinsic inhibitors that block growth in the immediate environment

of the glial scar. This combined approach may induce functional regeneration after CNS injury. Various strategies for blocking the inhibitory factors in SCI are described later in this chapter.

Humoral antibodies that promote remyelination bind to antigens on the surfaces of oligodendrocytes, suggesting that these antibodies might function through direct stimulation of the myelin-producing cells. An understanding of these mechanisms should open up significant new areas for the development of antibody-based therapeutics and perhaps also for small-molecule-based therapeutics and vaccines for induction of the reparative response.

28.4.2 Guiding Axons in the CNS

Most of the currently used methods of axonal regeneration approaches limit themselves to observe how axons elongate and migrate in response to signaling molecules presented on the substrate materials, or more recently, in response to different chemical and mechanical substrate properties. Many of these studies are encouraging in the hope of regenerating axons after disease or injury; however, numerous barriers remain. There is a need to optimize a permissive heterogeneous environment for axon elongation using tissue engineering approaches and a thorough understanding of the mechanical properties of the substrate, mechanotaxis, and both attractive and repulsive signaling mechanisms (Norman et al. 2009).

28.4.3 Cells Therapy for Regenerating CNS

Cell therapy for CNS disorders involves the use of cells of neural or non-neural origin to replace, repair, or enhance the function of the damaged nervous system and is usually achieved by transplantation of the cells, which are isolated and may be modified, e.g. by genetic engineering, when it may be referred to as gene therapy.

The olfactory ensheathing cells play an important role in CNS regeneration. In clinical trials, olfactory ensheathing cells have produced some of the most promising results including a functional recovery in humans following CNS injury (King-Robson 2011).

Glial support cells play an important role in the CNS and attempts have been made to transplant astrocytes, the major support cells in the CNS system by generating them from embryonic human glial precursor cells. There are differences in effects depending on how the astrocytes are generated. One study has provided a specific population of human astrocytes that appears to be particularly suitable for further development towards clinical application in treating the traumatically injured or diseased human CNS (Davies et al. 2011).

Neural stem cells migrate through the parenchyma along various routes in a precise, directed manner across great distances to injury sites in the CNS, where they might engage niches harboring local transiently-expressed reparative signals. Activation of endogenous neural stem cells is being considered along with stem cell transplantation for regeneration of the injured spinal cord.

Transplanted cells can be tracked in the CNS by using special labels and MRI. Labeling of human neural stem cells grown as neurospheres with magnetic nanoparticles was shown to not adversely affect survival, migration, and differentiation or alter neuronal electrophysiological characteristics (Guzman et al. 2007). Noninvasive cellular imaging has great potential for neurotransplantation as it enables real-time tracking of grafted cells as well as monitoring biodistribution and development (Walczak and Bulte 2007).

With the exception of autologous transplants, rejection is the main adverse event with cell transplants. Implanted stem cells may be tumorigenic. Development of a donor stem cell-derived glioneural brain tumor has been reported in a patient affected by the ataxia telangiectasia 4 years following repeated transplantations of fetal neural stem cells (Amariglio et al. 2009). Molecular and cytogenetic studies showed that the tumor was of non-host origin, suggesting it was derived from the transplanted cells.

28.4.4 Gene Therapy Approaches for Repair of CNS Injuries

Gene therapy has the potential to overcome many of the difficulties associated with the delivery of anti-scarring and neurotrophic substances to the site of an injury. Suitable and safe vectors for the delivery of genes need to be developed. Although there are several obstacles to making gene therapy practical and effective in humans, it has the potential to provide a new approach to the treatment of TBI (Shen et al. 2007).

28.4.5 Vaccines for Neuroregeneration

Inability of neurons and axons to regenerate following injury to the nervous system is due mostly to the presence of myelin and oligodendrocyte-related inhibitors of neurite outgrowth. A vaccine-based approach can be used to circumvent this issue and promote axonal regeneration and repair following traumatic injury (Ang et al. 2006). A vaccine against Nogo-66 (NgR), the common receptor for three myelin-associated inhibitors (Nogo-A, myelin-associated and oligodendrocyte myelin glycoprotein), has been shown to significantly improve functional recovery in rats subjected to spinal cord hemisection (Yu et al. 2008).

28.4.6 Role of Hyperbaric Oxygen in CNS Regeneration

Hyperbaric oxygen (HBO) therapy is therapeutic use of oxygen under greater than atmospheric pressure at sea level. Rationale for the neuroprotective effect of HBO in TBI is that it relieves hypoxia, improves the microcirculation, and relieves cerebral edema (Jain 2009a). HBO also mobilizes instrinsic stem cells and can contribute to regeneration following TBI.

28.4.7 Biomaterials for CNS Regeneration

Biomaterials can facilitate regeneration of the CNS as part of devices for targeted delivery of drugs or therapeutic proteins to the brain, as scaffolds for cell or tissue transplants, and to facilitate repair damaged neuronal pathways (Orive et al. 2009). Polyethylene glycol (PEG) and nanotechnology are providing the important methods for this regeneration and repair of the CNS.

28.4.7.1 Nanobiotechnology for Regeneration and Repair of the CNS

Nanotechnology is the creation and utilization of materials, devices, and systems through the control of matter on the nanometer (one billionth of a meter) scale. Nanobiotechnology is the application of nanotechnology in biotechnology leading to the development of nanomedicine (Jain 2012). Various nanomaterials have been designed to self-assemble into nanofibers and provide the framework for regeneration of nerve fibers in experimental studies on animal models of SCI. This enables greater control over material-cell interactions, which induce specific developmental processes and cellular responses, including differentiation, migration and outgrowth. In a nanofiber network, progenitor cells develop into neurons rather than astrocytes thus hindering the formation of scar tissue that hinders regeneration.

28.4.8 Pharmaceuticals to Facilitate Regeneration of the CNS

Several Drugs are being investigated or in development to enhance regeneration and repair of CNS injuries (Ibarra and Martiñón 2009). A classification of these is shown in Table 28.3.

28.4.8.1 Assessment of Potential of Drugs for Regenerating of CNS

Role of nitric oxide (NO)-cyclic guanosine-monophosphate (cGMP) transduction pathway in regulating axonal growth and neural migration has been demonstrated in

Table 28.3 Pharmaceutical approaches to facilitate regeneration of the CNS.

Agents that counteract the action of factors inhibiting regeneration following trauma
Anti-Nogo-A antibodies
Rho-ROCK inhibitors
Glial scar inhibitors
Local application of chondroitinase at the site of injury
Axon guidance molecules
Pharmacological modulation of the signal transduction pathways
Cyclic AMP-enhancers
Inhibitors of the phosphoinositide 3-kinase pathway
Inhibitors of inositol triphosphate receptor
Nitric oxide-cyclic guanosine-monophosphate transduction pathway
Agents that promote regeneration
Bone morphogenetic protein 7
Immunophilin ligands
Neurotrophic factors
Retinoic acid
Agents that improve remyelination
Fampridine
Monoclonal antibodies
Drugs that mobilize intrinsic stem cells
Drug combinations with devices and biological therapies

an invertebrate locust embryo model, which lacks the growth-inhibiting factors found in the CNS of higher vertebrates (Stern and Bicker 2008). Application of exogenous NO or cGMP promotes axonal regeneration, whereas scavenging NO or inhibition of soluble guanylyl cyclase delays regeneration, an effect that can be rescued by application of external cGMP. This embryo-culture system is a useful tool for studying effect of various drugs on CNS regeneration.

A model system of mouse entorhino-hippocampal slice cultures has been used to assess the potential of pharmacological treatments with compounds targeting signal transduction pathways to promote growth of entorhinal fibers after mechanical lesions across the lesion site to their target region in the dentate gyrus (Bonnici and Kapfhammer 2009). This method can be used for selection of promising compounds for further development to promote regeneration of CNS.

28.4.8.2 Pharmaceutical Manipulation of Stem Cells

Pharmaceutical manipulation of stem cells has the following aims: (1) to increase the number of multipotential cells; (2) to enhance the survival of implanted cells; (3) to influence the fate of specific endogenous multipotential cell populations; and (4) to influence the differentiation of stem cells.. Several biological and small molecules can enhance the in vivo and ex vivo regenerative properties of stem cells. Mozobil™ (Genzyme), a small molecule drug, increases the number of stem cells in the

circulating blood in human volunteers and enhances the effect of granulocyte colony stimulating factor. Lithium, a standard drug for manic depression with unknown mode of action, stimulates stem cells growing in culture to multiply faster, indicating that it could prompt stem cells in the brain to produce new cells to replace those that are damaged. The most important of the molecules for enhancing stem cells are neurotrophic factors.

28.4.8.3 Bone Morphogenetic Protein 7

Bone morphogenetic proteins (BMPs) are multifunctional growth factors that belong to the transforming growth factor-beta superfamily. BMPs regulate several crucial aspects of embryonic development and organogenesis. BMP-2/4 inhibits axonal regeneration and limits functional recovery following injury to the CNS, but BMP-2 is involved in the regeneration of peripheral nerves, and might function as a potential neurotrophic factor. BMP7, currently used in patients to treat non-neurological diseases, can induce neuroregeneration (Bani-Yaghoub et al. 2008). BMP may provide axon guidance cues, which may be used to force axons to grow in planned directions or patterns (Yaron and Zheng 2007).

28.4.8.4 Neurotrophic Factors

Neurotrophic factors (NTFs) are a class of naturally occurring protein growth factors that have multiple effects on the nervous system and potential therapeutic applications in neurological disorders. NTFs can be used for pharmacological control of endogenous neural stem cells (NSCs) to enhance brain repair and to improve integration of transplanted cells in the brain.

The most clinically advanced of NTFs, Glial Growth Factor 2 (GGF2), is a member of the neuregulin family of growth factors related to epidermal growth factor. The neuregulins bind to erbB receptors, which translate the growth factor signal to the cell and cause changes in cell growth, protein production and gene expression. The molecule was shown in published studies to stimulate remyelination in preclinical models of multiple sclerosis and to have a range of other effects in neural protection and repair. The neuroprotection and repair properties of neuregulins have led to promising results in a range of models of CNS injury. Research and development of neuregulin as a potential therapy in the treatment of TBI is in progress.

28.4.8.5 Retinoic Acid

Retinoic acid (RA) is involved in the induction of neural differentiation, motor axon outgrowth and neural patterning. Like other developmental molecules, RA continues to play a role after development has been completed. Elevated RA signaling in the adult triggers axon outgrowth with resulting nerve regeneration. RA is also

involved in the maintenance of the differentiated state of adult neurons, and disruption of RA signaling in the adult leads to the degeneration of motor neurons. RA could be used as a therapeutic molecule for the induction of axon regeneration (Maden 2007).

28.4.9 Role of Enriched Environments

Considerable published evidence shows that living in an enriched environment alters dendrites and synapses in the brains of adult rodents. A study on adult primates shows that the brain remains highly sensitive to experiential complexity, and living in standard laboratory housing may induce reversible dendritic spine and synapse decreases in brain regions important for cognition (Kozorovitskiy et al. 2005). Currently, enriched environment is considered to be the single most efficient plasticity and regeneration promoting paradigm (Nilsson and Pekny 2007). This has important implications for neurorehabilitation.

28.5 Clinical Aspects of CNS Regeneration

28.5.1 Assessment of Regeneration and Plasticity

Various neurophysiological methods, clinical neuropsychological assessments, and brain imaging studies can be used for assessing regeneration, recovery, and plasticity in the CNS.

Neurophysiological techniques. These techniques are useful for evaluating spontaneous recovery from damage and the therapeutic benefits of training, as well as other therapies. Transcranial magnetic stimulation has been used for this purpose.

Brain imaging techniques. Positron emission tomography and functional magnetic resonance imaging can be used to monitor the recovery and plasticity of the brain following injury. PET has been used to demonstrate changes in the activation of cortical and subcortical brain areas in response to altered spinothalamic and spinocerebellar input in paraplegic patients. These techniques have also been used to map clinically relevant plasticity after a stroke.

28.5.2 Neuroprotection and Neuroregeneration

It is generally believed that neuroprotection is required in the acute phase, and neuroregeneration is the long-term goal for restoring function following CNS injury, stroke, or neurodegenerative diseases. No clear-cut line of demarcation exists

between neuroprotection and neuroregeneration. The measures for achieving these overlap in accordance with the generally accepted concept that rehabilitation should start in the acute phase of neurologic disease. No acute phase is identifiable in some chronic neurologic disorders. For example, chronic traumatic encephalopathy may be the cumulative result of repeated cerebral concussions in athletes or soldiers and may have an insidious onset. Neuroregeneration efforts may not be successful without combination with neuroprotective strategies to counteract the progressive deterioration of neurologic function (Jain 2011). Cell transplants and gene therapy as methods for regeneration of the CNS may not be practical for application in the acute phase of injury, but both can have a long-term neuroprotective function.

28.5.3 Management of Spinal Cord Injury

Significant spontaneous functional recovery may occur over several years following incomplete SCI. Possible mechanisms involved are synaptic plasticity in pre-existing pathways and the formation of new circuits through collateral sprouting of lesioned and unlesioned fibers. Some evidence shows that plasticity can be facilitated by activity or experimental manipulations. These studies form a basis for the development of new rehabilitation approaches for SCI.

Several neuroregenerative approaches that are being pursued for acute SCI with complete functional transection are shown in Table 28.4.

28.5.3.1 Antagonism of Inhibitors of Regeneration Following SCI

Several inhibitors of regeneration of spinal cord following SCI have been identified and strategies to counteract most of these factors have been developed. Chondroitin sulfate proteoglycans (CSPGs) are a major class of axon growth inhibitors that are up-regulated after SCI and contribute to regenerative failure. Degradation of CSPGs after SCI by application of chondroitinase at the site of injury may promote regeneration of corticospinal tract axons. This approach has been effective in animal models of SCI and has potential for the treatment of human SCI. Therapeutic strategies aimed at inhibition of collagen matrix formation in brain and spinal cord lesions promote axonal regeneration and functional recovery (Klapka and Muller 2006).

Chondroitin sulfate proteoglycans (CSPGs) are a major class of axon growth inhibitors that are up-regulated after spinal cord injury (SCI) and contribute to regenerative failure. Chondroitinase ABC (chABC) digests glycosaminoglycan chains on CSPGs and can counteract CSPG-mediated inhibition, but it loses its enzymatic activity rapidly at 37 °C, requiring the use of repeated injections or local infusions for prolonged periods. A thermostabilized chABC and a system for its sustained local delivery have been developed to overcome this problem (Lee et al. 2010). Animals treated with sustained delivery of thermostabilized chABC in combination with neurotrophin-3 showed enhanced growth sensory axons and sprouting of serotonergic fibers.

Table 28.4 Strategies for neuroregenerative in acute SCI

Strategy	Rationale
Neuroprotection	To counteract the progression of damage following initial injury in order to facilitate regeneration
Careful modulation of the inflammatory response following trauma	Usually beneficial to recovery of function but may be detrimental in some situations
Antagonists of inhibitors of regeneration	To facilitate regeneration (see text for details)
Restoration of neurotransmission	Based on the concept that the dysfunctions that occur after SCI are primarily due to damage to neurotransmission (Xu and Onifer 2009)
Cell transplantation	Replacement of damaged tissue
Neurotrophic factors	Enhance axonal plasticity and regeneration after SCI (Hollis and Tuszynski 2011)
Peripheral nerve transplantation	Bridging the disrupted segment of spinal cord to provide a pathway for regenerating nerve fibers
Nanofiber struts with or without stem cells	Scaffolds to promote regeneration
Manipulation of the extracellular matrix composition	To provide a supportive environment for sprouting and regenerating neurons and reduces glial scarring
Synthetic/biodegradable gel/polymer implants	To serve as substrates for neurite outgrowth and synapse formation
Transfer of acidic fibroblast growth factor (aFGF) gene with adeno-associated virus	aFGF was shown to improve functional recovery in spinal cord-contused rats (Huang et al. 2011)
Vaccines	To stimulate nerve regeneration
Anti-Nogo-A antibodies for neutralizing the inhibitory effect of Nogo-A	These antibodies have been shown to enhance fiber growth, regeneration, and functional recovery in primate models of SCI (Buchli et al. 2007)
Forced upregulation of mTOR (mammalian target of rapamycin) activity by deletion of PTEN (phosphatase and tensin homolog), a negative regulator of mTOR to enable successful regeneration of corticospinal axons past a spinal cord lesion.	An experimental study showed that the regrowth potential of corticospinal tract axons was lost in the fully grown mouse and this was accompanied by a downregulation of mTOR activity in corticospinal neurons, which further diminished following axonal injury (Liu et al. 2010)

Knowledge of axon regeneration inhibitors provides new opportunities for therapeutic development of counteracting these inhibitors for spinal SCI (Xie and Zheng 2008). Neurite outgrowth inhibitors and their receptors can be blocked with antibodies or peptides to facilitate regeneration. Some of the specific antagonists are commercially available for experimental investigations. Administration of an inhibitor of Sema3A, a guidance molecule that is expressed by the fibroblast component of the scar tissue, and leads to multiple beneficial effects including enhanced regenerative response from axons (Kaneko et al. 2006).

Rho-kinase (ROCK) is a serine/threonine kinase and one of the major downstream effectors of the small GTPase Rho. The Rho-ROCK pathway is involved in many aspects of neuronal functions including neurite outgrowth and retraction. The Rho-ROCK pathway is an attractive target for the development of drugs for treating CNS disorders, since it has been recently revealed that this pathway is closely related to the pathogenesis of several CNS disorders such as SCI. The effects of regeneration inhibitors are reversed by blockade of the Rho-ROCK pathway in vitro, and the inhibition of this pathway promotes axonal regeneration and functional recovery in the injured CNS in vivo (Kubo et al. 2008).

Eph receptor tyrosine kinase family and their ligands inhibit axonal regeneration following CNS injury. Antagonism of one of these, EphA4 is a potential therapy to promote recovery from SCI based on the demonstration that axonal regeneration following SCI is promoted in an EphA4 knockout animal (Goldshmit et al. 2011). Further studies by these authors in animal models of SCI provide definite evidence that soluble inhibitors of EphA4 function offer considerable therapeutic potential for the treatment of SCI.

Cortical gene expression profiling studies with microarrays in the rat have shown that anti-scarring treatment attenuates SCI-triggered transcriptional changes of genes related to inhibition of axon growth and impairment of cell survival, while upregulating the expression of genes associated with axon outgrowth, cell protection, and neural development (Kruse et al. 2011). This treatment not only modifies the local environment impeding spinal cord regeneration by reduction of fibrous scarring in the injured spinal cord, but, in addition, strikingly changes the activity of cortical neurons that is favorable for axonal regeneration.

28.5.3.2 Cell Transplantation for SCI

Considerable advances have been made during the past decade in devising and evaluating axon regeneration strategies based on cell transplants for SCI patients. Table 28.5 lists various types of cells used for this purpose.

Grafted cultured keratinocytes secrete growth factor scan induce growth of cell that have survived as well as neural differentiation of stem cells surrounding the injured spinal cord, leading to functional recovery (Inoue et al. 2011). A chitosan conduit loaded with bone marrow stem cells (BMSCs) was shown to significantly reduce the spinal cord cavity volume at the injured site in adult rats and the results suggest that it may become a promising approach to the repair of SCI in humans (Chen et al. 2011). In another study, Rats with thoracic SCI could walk with weight bearing and showed recovered motor evoked potentials following transplanted of neurospheres (NS) derived from BMSCs (Suzuki et al. 2011). Histological analysis of spinal cords showed neuronal or axonal sproutings, which were replaced by host cells. Also, transplanted BMSCs-NS expressed neuronal lineage biomarkers.

A study has directed neural differentiation of murine iPS cells and examined their therapeutic potential in a mouse SCI model (Tsuji et al. 2010). Safe iPS-derived neurospheres, which had been pre-evaluated as nontumorigenic, were transplanted

Table 28.5 Types of cells used for transplantation in SCI

Cell type	Rationale for use
Autoimmune T cells against CNS myelin-associated peptide	Neuroprotective effect in experimental models by reducing the spread of damage and promotion of recovery in injured rat spinal cord
Glial cells: progenitor-derived astrocytes or cultured	Glia-depleted areas of the CNS can be reconstituted by introducing glial cells
Olfactory ensheathing glial cells	Facilitate regeneration
Keratinocytes	Secrete growth factors
Embryonic stem cells (ESCs)	To replace the lost neurons and supporting cells
Autologous bone marrow stem cells	A clinical trial in patients with chronic complete SCI produced slight neurologic improvement (Deda et al. 2008)
Neural stem cells (NSCs)	Generated from ESCs and can be developed into motor neurons
Induced pluripotent stem (iPS) cells	iPS clone-derived NSCs may be a promising cell source for transplantation therapy for SCI

into the spinal cord after contusive injury. The neurospheres produced remyelination and induced axonal regrowth promoting locomotor function recovery. These results show that iPS clone-derived NSCs cells may be a promising cell source for transplantation therapy for SCI.

Progress in stem cell biology has made it feasible to induce the regeneration of injured axons after SCI in experimental animals by transplanting neural stem cells (NSCs) generated from the ESCs, which can be developed into motor neurons by using special techniques and culture media with growth factors. In an open trial, intravenous injection of autologous bone marrow cells in conjunction with the administration of granulocyte macrophage-colony stimulating factor led to improvement in patients with complete spinal cord injury (Park et al. 2005). Cell transplantation alone may not suffice for regeneration of the spinal cord and may need to be combined with other methods such as neurotrophic factors, blocking of inhibitors of neural regeneration, and modulation of inflammatory response following injury (Ronsyn et al. 2008).

Transplantation of autologous bone marrow-derived mesenchymal stem cells (MSC), expanded ex vivo, has been tested in a clinical trial on SCI patients (Pal et al. 2009). After quality control and characterization for cell surface markers, MSCs were administered to the patients via lumbar puncture. Safety of the procedure was demonstrated by 1–3 years of follow-up. No recovery of neurological deficit was reported but further trials with higher doses and different routes of administration were suggested in order to demonstrate the recovery/efficacy if any, in SCI patients. In addition to clinical trials, therapeutic use of stem cells in SCI with unverified claims of recovery of paraplegia is being reported from countries without strict regulatory controls.

Several studies have shown that transplantation of ESCs to replace the lost neurons and other supporting cells into adult rats that were partially paralyzed through spinal cord damage led to some recovery of spinal cord function. Introduction of stem cells into the cerebrospinal fluid via lumbar puncture is as effective as direct injection into the spinal cord. A clinical trial showed that subarachnoid placement of stem cells is safe with no long-term adverse effects (Mehta et al. 2008).

A spinal contusion injury model in the rat has been used to assess the efficacy of hESC-derived oligodendrocyte progenitor cells (OPCs) for cervical SCI (Sharp et al. 2010). hESC-derived OPC transplants attenuated lesion pathogenesis and improved recovery of forelimb function. Histological effects of transplantation included robust white and gray matter sparing at the injury epicenter, and in particular, preservation of motor neurons that correlated with movement recovery. These findings further an understanding of the histopathology and functional outcomes of cervical SCI, define potential therapeutic targets, and support the use of these cells as a treatment for cervical SCI. Based on this, Geron Corporation's GRNOPC1 started a clinical trial. A special device was used to inject hESCs into the spinal cords of patients with complete paraplegia due to SCI to determine if the procedure is safe and also if there will be any signs of recovery of function. Although safety was demonstrated, the company discontinued further development due to economic reasons.

28.5.3.3 Polyethylene Glycol for Repair of Nerve Fibers in SCI

Polyethylene glycol (PEG) can reunite and fuse transected cell processes and seal anatomical disruptions in cell membranes produced by mechanical injury. Topical application of PEG has been used for repair of spinal axons after severe, standardized SCI in experimental animals. PEG reduces both necrosis and apoptosis through two distinct yet synergistic pathways: repair of disrupted plasma membranes and protection of mitochondria through direct interaction (Luo and Shi 2007). An intravenous preparation of PEG has been used safely in a trial on dogs with SCI with some recovery of paraplegia (Laverty et al. 2004). Safety of PEG for human use is already established and it is feasible to translate this technique into application for human SCI. However, further studies have shown that although PEG protects key axonal cytoskeletal proteins after SCI along with axonal preservation, the modest extent of locomotor recovery after treatment with PEG may not be sufficient for clinical use as a single treatment (Baptiste et al. 2009).

28.5.3.4 Fampridine for SCI

Fampridine-SR is a sustained-release tablet form of the K^+ channel-blocking compound 4-aminopyridine that has been shown to restore conduction in focally demyelinated axons, to enhance synaptic transmission in many types of neurons and to potentiate muscle contraction. Most of the clinical development is focused on

multiple sclerosis but it also includes trials on SCI. A phase II double-blind, randomized, placebo-controlled, parallel-group clinical trial of sustained-release fampridine (25 mg twice daily) in chronic SCI showed significant improvement in Subject Global Impression, and potential benefit on spasticity (Cardenas et al. 2007). Phase III clinical results of Fampridine were positive and the FDA accepted the Fampridine-SR New Drug Application (NDA) for filing.

28.5.3.5 Monoclonal Antibodies for Repair in SCI

Natural autoreactive Ig monoclonal antibodies (MAbs) bind surface antigens on specific CNS cells, activating intracellular repair-promoting signals. Ig MAbs that bind to neurons stimulate neurite outgrowth and prevent death of neurons and have potential application for repair in neuron-damaging diseases, such as SCI (Wright et al. 2009). Recombinant remyelination-promoting Ig MAbs have been produced and undergone a phase 1 clinical trial after toxicology studies. Whereas Fampridine-SR (Accorda Therapeutics) may improve walking ability following SCI by pharmacologically compensating for myelin loss in some axons, MAbs may actually replace the lost myelin, which may provide additional benefit.

28.5.3.6 Role of RNAi and Other Knockdown Technologies in Regeneration Following SCI

Following SCI, there are numerous changes in gene expression that appear to contribute to either neurodegeneration or reparative processes. RNAi, ribozymes, and antisense technologies are used to suppress these. In animal experimental studies, several genes including the small GTPase Rab13 and actin-binding protein Coronin 1b, show significantly increased mRNA expression after SCI and this enhances neurite outgrowth. RNAi gene silencing for Coronin 1b or Rab13 in NGF-treated PC-12 cells markedly reduces neurite outgrowth (Di Giovanni et al. 2005). Modulation of these proteins may provide novel targets for facilitating restorative processes after SCI.

A target for preclinical drug development is the Nogo pathway, which plays a key role in preventing regeneration of nerves after injury, such as SCI. An RNAi therapeutic that inhibits this pathway could potentially reduce or prevent paralysis caused by such injuries.

Another knockdown technology uses deoxyribozymes, which are catalytic DNA molecules. Deoxyribozyme against xylosyltransferase (XT-1) mRNA not only prevents glycosylation of proteoglycans but also avoids the assembly of their core protein into the extracellular matrix. Thus it alters the inhibitory nature of the scar and promotes axonal growth in the injured spinal cord (Grimpe 2011). The immunological status of deoxyribozyme is not known and it does not penetrate the blood-brain or blood-spinal cord barrier. If used cautiously with an appropriate delivery technology, deoxyribozyme technology has the potential to become a major in CNS regeneration.

28.5.3.7 Physical Methods for Promoting Regeneration in SCI Patients

Several physical methods have been applied to promote regeneration in SCI. One of these is functional electrical stimulation (FES). An electric field oriented in the direction of a damaged neural fiber can enhance axonal regrowth. The technique of oscillating field stimulation applied to SCI has been shown to promote bidirectional regeneration in the injured nerve fibers (Hamid and Hayek 2008).

Neuroprostheses are machines designed to artificially restore lost neurologic function. Recent emphasis is on development of neuroprosthetic devices that utilize the information recorded directly from the CNS. Development of neurorobots for simple walking movements may require elaborate systems for timed interaction between sensory input and rhythmic motor output programs. There is some evidence that continuous CNS machine interaction and repeated activation facilities regeneration of the injured spinal cord and development of plasticity.

28.5.3.8 Combined Approaches to SCI

Following complete spinal cord transection that removes all supraspinal inputs in adult rats, spinal locomotion was shown to emerge from a combination of functional electrical stimulation and pharmacological approaches (Courtine et al. 2009). The development of central pattern-generating capability and the ability of these spinal circuits to use sensory afferent input to control stepping provide a strategy by which individuals with SCI could regain substantial levels of motor control.

28.5.4 Management of Traumatic Brain Injury

TBI or neurosurgical procedures may cause extensive loss of cerebral parenchyma. Reconstruction and regeneration is desirable, not only to replace the lost brain substance, but also to restore lost function and prevent formation of scar tissue. However, no clinically effective method is available as yet.

28.5.4.1 Cell Therapy for TBI

Cell therapy is expected to play an important role in the repair of TBI (Jain 2009b). It is important that cells are transplanted into an environment that is favorable for extended survival and integration within the host tissue (Tate et al. 2009). Extracellular matrix proteins such as fibronectin and laminin are involved in neural development and may mediate subsequent cell signaling events. Enhanced cell survival was demonstrated following transplantation of a NSC construct containing laminin-based scaffold into the traumatically injured mouse brain.

Stem cell-based cellular replacement strategies have a potential therapeutic role following TBI, but the mechanism by which stem cells produce their effect (e.g. via integration into surviving neuronal circuits, local neurotrophic support, or modification of the local microenvironment to enhance endogenous regeneration and neuroprotection) remains to be assessed further (Maegele and Schaefer 2008). One of the functions of stem cells is to decrease inflammation whereby regeneration can be facilitated.

NSCs transplanted directly into the injured brain were shown to survive, differentiate into neurons and promote functional recovery in a rat model of TBI (Ma et al. 2011a). There was an increase in the expression of SYP and GAP43 in the injured brain of NSC-transplanted rats, suggesting it as one of the mechanisms underlying the improved functional recovery.

A clinical trial to gauge the safety and potential of treating children suffering from TBI using hematopoietic stem cells derived from their own bone marrow started in 2008 (Harting et al. 2008). The clinical trial is based on laboratory and animal research indicating that HSCs can migrate to an injured area of the brain, differentiate into new neurons and support cells, and induce brain repair.

28.5.4.2 Gene Therapy for TBI

Currently available information from preclinical studies reveals that there are several gene targets with therapeutic potentials in TBI and vectors that can be used to deliver the candidate genes. However, there are difficulties in translating these techniques into effective gene therapy in humans. Examples of some of these studies are given in the following paragraphs.

NSCs genetically modified to encode BDNF gene (BDNF/NSCs) have been shown to significantly improve neurological motor function on selected behavioral tests following transplantation into brains of rats with TBI (Ma et al. 2011b). The number of surviving engrafted cells and the proportion of engrafted cells with a neuronal phenotype were significantly greater in BDNF/NSCs than in naive NSCs-transplanted rats that served as controls.

Neuronal regeneration can be induced by transgenic integrin expression. Integrins are nerve cell receptors that have been linked to the growth of nerve cells. Nerve cells taken from developing animals typically have high levels of integrins compared with those taken from adult animals. In experimental studies, the regenerative performance of adult neurons can be restored to that of young neurons by the gene transfer-mediated expression of a single alpha-integrin, which has the potential to be developed into a gene therapy approach to regeneration. The therapy would be based on the modification of integrin genes with a type of "switch" controlled by a drug. Physicians would then inject the modified genes into damaged area of the brain. A recombinant adenovirus vector expressing Bcl-2 fusion protein can suppress apoptosis and promote cell survival in experimental TBI in rats (Yang et al. 2006).

28.5.4.3 Nanomaterial Scaffolds for Repair of TBI

The peptide nanofiber scaffold is an effective technology for tissue repair and restoration and is a promising treatment for TBI. This peptide nanofiber scaffold has several advantages over currently available polymer biomaterials. The network of nanofibers is similar in scale to the native extracellular matrix and, thus, provides an environment for cell growth, migration, and differentiation. This peptide disintegrates and is immunologically inert. Self-assembling peptide nanofiber scaffold may help to reconstruct the acutely injured brain and reduce the glial reaction and inflammation in the surrounding brain tissue (Guo et al. 2009; Webber et al. 2010). This creates a permissive environment for axons, not only to regenerate through the site of an acute injury, but also to knit the brain tissue together.

Challenges of using a tissue engineering approach for regeneration in TBI include a complex environment and variables that are difficult to assess. For optimal benefit, the brain should be in a condition that minimizes immune response, inflammation and rejection of the grafted material. Tissue engineering, using a bioactive scaffold counters some of the hostile factors and facilitates integration of donor cells into the brain, but transplantation of a combination biologic construct to the brain has not yet been successfully translated into clinical use (Stabenfeldt et al. 2011).

The next generation of tissue engineering scaffolds for TBI may incorporate nanoscale surface feature dimensions, which mimic natural neural tissue. Nanomaterials can enhance desirable neural cell activity while minimizing unwanted astrocyte reactivity. Composite materials with zinc oxide nanoparticles embedded into a polymer matrix can provide an electrical stimulus when mechanically deformed through ultrasound, which can act as a cue for neural tissue regeneration (Seil and Webster 2010).

28.5.4.4 Neuroregeneration in Chronic Traumatic Encephalopathy

Chronic traumatic encephalopathy (CTE) is the term used for neuropathological changes consistent with long-term repetitive concussive brain injury traditionally seen in football players and boxers, but now in US soldiers returning from Iraq and Afghanistan wars. Clinical manifestations include cerebral dysfunction with cognitive impairments and neurobehavioral disturbances, which may progress to dementia as tearing of neuronal connections (axonal shearing) disconnects or impairs cortical and thalamic circuits. Neuropathological changes in the brain are those of a tauopathy with neurofibrillary deposits. The cumulative effect of repeated concussions is different from acute TBI and resembles more the course of a neurodegenerative disease. Apart from avoidance of trauma, neuroprotective and neuroregenerative measures may be considered for CTE, which may be similar to those for neurodegenerative disorders. HBO has been found to be useful for treatment of CTE due to blast injury (Harch et al. 2009). Pharmacological strategies include development of anti-tau drugs to clear tau deposits.

28.6 Concluding Remarks and Future Prospects

Considerable knowledge has been gained about regeneration in the CNS in experimental animals by studying factors inhibiting neurite outgrowth and using substances to inhibit these. Worldwide, research on regeneration in SCI has historically preceded that in TBI and there has been more activity in the former. Currently TBI has gained priority in research in the US due to a large number of injured soldiers from various wars and greater recognition of CTE in soldiers and athletes. However, translation of these into application in human CNS injury has only limited success.

Although many strategies are being pursued currently, several challenges still remain. Areas that are promising for future research in regeneration following CNS trauma are:

- Cell therapies, particularly by use of stem cells.
- Use of biodegradable materials such as polymer nanofibers to provide support and a favorable environment for CNS regeneration.
- Strategies for inhibiting signaling mechanisms that hinder regeneration.
- Efforts continue to develop more effective anti-scarring treatment to promote regeneration in SCI.
- Focus on functional recovery rather than mere structural restoration.
- Combination of multiple methods such as use of biological therapies and pharmaceuticals.

References

Amariglio N, Hirshberg A, Scheithauer BW et al (2009) Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med 6(2):e1000029

Ang BT, Xu G, Xiao ZC (2006) Therapeutic vaccination for central nervous system repair. Clin Exp Pharmacol Physiol 33:541–5

Atalay B, Bavbek M, Ozen O et al (2008) Nogo-A inhibitory peptide (NEP1-40) increases pan-cadherin expression following mild cortical contusion injury in rats. Turk Neurosurg 18:356–65

Bani-Yaghoub M, Tremblay RG, Ajji A et al (2008) Neuroregenerative strategies in the brain: emerging significance of bone morphogenetic protein 7 (BMP7). Biochem Cell Biol 86:361–9

Baptiste DC, Austin JW, Zhao W, Nahirny A, Sugita S, Fehlings MG (2009) Systemic polyethylene glycol promotes neurological recovery and tissue sparing in rats after cervical spinal cord injury. J Neuropathol Exp Neurol 68:661–76

Bonnici B, Kapfhammer JP (2009) Modulators of signal transduction pathways can promote axonal regeneration in entorhino-hippocampal slice cultures. Eur J Pharmacol 612:35–40

Buchli AD, Rouiller E, Mueller R, Dietz V, Schwab ME (2007) Repair of the injured spinal cord. A joint approach of basic and clinical research. Neurodegener Dis 4:51–6

Buss A, Pech K, Kakulas BA et al (2009) NG2 and phosphacan are present in the astroglial scar after human traumatic spinal cord injury. BMC Neurol 9:32. doi:10.1186/1471-2377-9-32

Cardenas DD, Ditunno J, Graziani V et al (2007) Phase 2 trial of sustained-release fampridine in chronic spinal cord injury. Spinal Cord 45:158–68

Chen X, Yang Y, Yao J et al (2011) Bone marrow stromal cells-loaded chitosan conduits promote repair of complete transection injury in rat spinal cord. J Mater Sci Mater Med 22:2347–56

Cotman CW, Scheff SW (1979) Compensatory synapse growth in aged animals after neuronal death. Mech Ageing Dev 9:103–17
Courtine G, Gerasimenko Y, van den Brand R et al (2009) Transformation of nonfunctional spinal circuits into functional states after the loss of brain input. Nat Neurosci 12:1333–42
Davies SJA, Shih C-H, Noble M et al (2011) Transplantation of specific human astrocytes promotes functional recovery after spinal cord injury. PLoS One 6(3):e17328
Deda H, Inci M, Kurekci A et al (2008) Treatment of chronic spinal cord injured patients with autologous bone marrow-derived hematopoietic stem cell transplantation: 1-year follow-up. Cytotherapy 10:565–74
del Rio-Hortega P (1932) Microglia. In: Penfield W (ed) Cytology and cellular pathology of the nervous system, vol 2. Paul Hoeber, New York, pp 481–534
Di Giovanni S, De Biase A, Yakovlev A et al (2005) In vivo and in vitro characterization of novel neuronal plasticity factors identified following spinal cord injury. J Biol Chem 280:2084–91
Filbin MT (2008) PirB, a second receptor for the myelin inhibitors of axonal regeneration Nogo66, MAG, and OMgp: implications for regeneration in vivo. Neuron 60:740–2
Goldshmit Y, Spanevello MD, Tajouri S et al (2011) EphA4 Blockers promote axonal regeneration and functional recovery following spinal cord injury in mice. PLoS One 6(9):e24636
Goldstein K (1931) Uber die plastitat des organismus auf grund von erfahrungen am nervenkranken menschen. In: Bethe A (ed) Handbuch der normalen und pathologischen physiologie. Springer, Berlin, pp 1131–74
Grimpe B (2011) Deoxyribozymes: new therapeutics to treat central nervous system disorders. Front Mol Neurosci 4:25
Guo J, Leung KK, Su H et al (2009) Self-assembling peptide nanofiber scaffold promotes the reconstruction of acutely injured brain. Nanomedicine 5:345–51
Guzman R, Uchida N, Bliss TM et al (2007) Long-term monitoring of transplanted human neural stem cells in developmental and pathological contexts with MRI. Proc Natl Acad Sci USA 104:10211–6
Hamid S, Hayek R (2008) Role of electrical stimulation for rehabilitation and regeneration after spinal cord injury: an overview. Eur Spine J 17:1256–69
Harch PG, Fogarty EF, Staab PK, Van Meter K (2009) Low pressure hyperbaric oxygen therapy and SPECT brain imaging in the treatment of blast-induced chronic traumatic brain injury (post-concussion syndrome) and post traumatic stress disorder: a case report. Cases J 2:6538
Harting MT, Baumgartner JE, Worth LL et al (2008) Cell therapies for traumatic brain injury. Neurosurg Focus 24:E18
Heinrich C, Blum R, Gascon S et al (2010) Directing astroglia from the cerebral cortex into subtype specific functional neurons. PLoS Biol 8(5):e1000373
Hollis ER 2nd, Tuszynski MH (2011) Neurotrophins: potential therapeutic tools for the treatment of spinal cord injury. Neurotherapeutics 8(4):694–703
Huang WC, Kuo HS, Tsai MJ et al (2011) Adeno-associated virus-mediated human acidic fibroblast growth factor expression promotes functional recovery of spinal cord-contused rats. J Gene Med 13:283–9
Ibarra A, Martiñón S (2009) Pharmacological approaches to induce neuroregeneration in spinal cord injury: an overview. Curr Drug Discov Technol 6:82–90
Inoue H, Takenaga M, Ohta Y et al (2011) Improvement of hind-limb paralysis following traumatic spinal cord injury in rats by grafting normal human keratinocytes: new cell-therapy strategy for nerve regeneration. J Artif Organs 14(4):375–80
Jain KK (2009a) Textbook of hyperbaric medicine, 5th edn. Hogrefe, Göttingen
Jain KK (2009b) Cell therapy for CNS trauma. Mol Biotechnol 42:367–76
Jain KK (2011) Handbook of neuroprotection. Springer – Humana Press, Totowa
Jain KK (2012) The handbook of nanomedicine, 2nd edn. Springer – Humana Press, Totowa
Kaneko S, Iwanami A, Nakamura M et al (2006) A selective Sema3A inhibitor enhances regenerative responses and functional recovery of the injured spinal cord. Nat Med 2:380–9
King-Robson J (2011) Encouraging regeneration in the central nervous system: is there a role for olfactory ensheathing cells? Neurosci Res 69:263–75

Klapka N, Muller HW (2006) Collagen matrix in spinal cord injury. J Neurotrauma 23:422–35
Kozorovitskiy Y, Gross CG, Kopil C et al (2005) Experience induces structural and biochemical changes in the adult primate brain. Proc Natl Acad Sci USA 102:17478–82
Kruse F, Bosse F, Vogelaar CF et al (2011) Cortical gene expression in spinal cord injury and repair: insight into the functional complexity of the neural regeneration program. Front Mol Neurosci 4:26
Kubo T, Yamaguchi A, Iwata N, Yamashita T (2008) The therapeutic effects of Rho-ROCK inhibitors on CNS disorders. Ther Clin Risk Manag 4:605–15
Laverty PH, Leskovar A, Breur GT et al (2004) A preliminary study of intravenous surfactants in paraplegic dogs: polymer therapy in canine clinical SCI. J Neurotrauma 21:1767–77
Lee H, McKeon RJ, Bellamkonda RV (2010) Sustained delivery of thermostabilized chABC enhances axonal sprouting and functional recovery after spinal cord injury. Proc Natl Acad Sci USA 107:3340–5
Liu K, Lu Y, Lee JK et al. (2010) PTEN deletion enhances the regenerative ability of adult corticospinal neurons. Nat Neurosci 13:1075–81
Luo J, Shi R (2007) Polyethylene glycol inhibits apoptotic cell death following traumatic spinal cord injury. Brain Res 1155:10–6
Ma H, Yu B, Kong L et al (2011a) Transplantation of neural stem cells enhances expression of synaptic protein and promotes functional recovery in a rat model of traumatic brain injury. Mol Med Rep 4:849–56
Ma H, Yu B, Kong L et al (2011b) Neural stem cells over-expressing brain-derived neurotrophic factor (BDNF) stimulate synaptic protein expression and promote functional recovery following transplantation in Rat model of traumatic brain injury. Neurochem Res 37(1):69–83
Maden M (2007) Retinoic acid in the development, regeneration and maintenance of the nervous system. Nat Rev Neurosci 8:755–65
Maegele M, Schaefer U (2008) Stem cell-based cellular replacement strategies following traumatic brain injury (TBI). Minim Invasive Ther Allied Technol 17:119–31
Mehta T, Feroz A, Thakkar U et al (2008) Subarachnoid placement of stem cells in neurological disorders. Transplant Proc 40:1145–7
Mitchell SW (1872) Injuries of nerves. JB Lippincott & Co, Philadelphia
Neumann S, Woolf CJ (2000) Regeneration of dorsal column fibers into and beyond the lesion site following spinal cord injury. Neuron 23:83–91
Nilsson M, Pekny M (2007) Enriched environment and astrocytes in central nervous system regeneration. J Rehabil Med 39:345–52
Norman LL, Stroka K, Aranda-Espinoza H (2009) Guiding axons in the central nervous system: a tissue engineering approach. Tissue Eng Part B Rev 15:291–305
Orive G, Anitua E, Pedraz JL, Emerich DF (2009) Biomaterials for promoting brain protection, repair and regeneration. Nat Rev Neurosci 10:682–92
Pal R, Venkataraman NK, Jan M et al (2009) Ex vivo-expanded autologous bone marrow-derived mesenchymal stromal cells in human spinal cord injury/paraplegia: a pilot clinical study. Cytotherapy 11:897–911
Park HC, Shim YS, Ha Y et al (2005) Treatment of complete spinal cord injury patients by autologous bone marrow cell transplantation and administration of granulocyte-macrophage colony stimulating factor. Tissue Eng 11:913–22
Ramon y Cajal S (1959) Degeneration and regeneration in the nervous system (trans: May RM). Haffner, New York
Ronsyn MW, Berneman ZN, Van Tendeloo VF, Jorens PG, Ponsaerts P (2008) Can cell therapy heal a spinal cord injury? Spinal Cord 46:532–9
Seil JT, Webster TJ (2010) Electrically active nanomaterials as improved neural tissue regeneration scaffolds. Wiley Interdiscip Rev Nanomed Nanobiotechnol 2:635–47
Sharp J, Frame J, Siegenthaler M et al (2010) Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. Stem Cells 28:152–63
Shen F, Wen L, Yang X, Liu W (2007) The potential application of gene therapy in the treatment of traumatic brain injury. Neurosurg Rev 30:291–8

Stabenfeldt SE, Irons HR, Laplaca MC (2011) Stem cells and bioactive scaffolds as a treatment for traumatic brain injury. Curr Stem Cell Res Ther 6:208–20
Stern M, Bicker G (2008) Nitric oxide regulates axonal regeneration in an insect embryonic CNS. Dev Neurobiol 68:295–308
Suzuki H, Taguchi T, Kato Y et al (2011) Transplantation of neurospheres derived from bone marrow stromal cells promotes neurological recovery in rats with spinal cord injury. Med Mol Morphol 44:131–8
Tate CC, Shear DA, Tate MC, Archer DR, Stein DG, LaPlaca MC (2009) Laminin and fibronectin scaffolds enhance neural stem cell transplantation into the injured brain. J Tissue Eng Regen Med 3:208–17
Thompson WG (1890) Successful brain grafting. N Y Med J 51:701
Tsuji O, Miura K, Okada Y et al (2010) Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. Proc Natl Acad Sci USA 107:12704–9
Walczak P, Bulte JW (2007) The role of noninvasive cellular imaging in developing cell-based therapies for neurodegenerative disorders. Neurodegener Dis 4:306–13
Walmsley AR, Mir AK (2007) Targeting the Nogo-A signalling pathway to promote recovery following acute CNS injury. Curr Pharm Des 13:2470–84
Webber MJ, Kessler JA, Stupp SI (2010) Emerging peptide nanomedicine to regenerate tissues and organs. J Intern Med 267:71–88
Wright BR, Warrington AE, Edberg DD, Rodriguez M (2009) Cellular mechanisms of central nervous system repair by natural autoreactive monoclonal antibodies. Arch Neurol 66:1456–9
Xie F, Zheng B (2008) White matter inhibitors in CNS axon regeneration failure. Exp Neurol 209:302–12
Xu XM, Onifer SM (2009) Transplantation-mediated strategies to promote axonal regeneration following spinal cord injury. Respir Physiol Neurobiol 169:171–82
Yang XF, Zheng XS, Liu WG, Feng JF (2006) Bcl-2 gene therapy for apoptosis following traumatic brain injury. Chin J Traumatol 9:276–81
Yaron A, Zheng B (2007) Navigating their way to the clinic: emerging roles for axon guidance molecules in neurological disorders and injury. Dev Neurobiol 67:1216–31
York GK, Steinberg DA (1994) Hughling Jackson's theory of cerebral localization. J Hist Neurosci 3:153–68
Yu P, Huang L, Zou J et al (2008) Immunization with recombinant Nogo-66 receptor (NgR) promotes axonal regeneration and recovery of function after spinal cord injury in rats. Neurobiol Dis 32:535–42

Chapter 29
Regenerative Therapies for the Ocular Surface

Geeta K. Vemuganti, Virender S. Sangwan, Indumathi Mariappan, and Dorairajan Balasubramanian

Abstract Integrity of ocular surface depends on adequate tear film and stability of the surface epithelium consisting of two specialized phenotypically different epithelial cells – the central transparent corneal epithelium and the peripheral conjunctival cells, separated by a more specialized transition zone, called the limbus. Similar to the epithelial regeneration in other parts of the body, the corneal epithelium is regenerated from the stem cells located in limbus. Severe chemical burns and other diseases can cause damage to the limbus, resulting in a condition called Limbal Stem Cell Deficiency (LSCD). Effective therapeutic modalities for this vision-threatening condition include use of human amniotic membrane, replenishing the stores of limbal stem cells by limbal transplantation. However last decade has witnessed the use of ex-vivo expanded sheet of limbal epithelial cells for ocular surface reconstruction in such cases. Our group has established a simple, feeder-cell free, cost-effective way of culturing the corneal epithelium from limbal tissues within 2 weeks, using human amniotic membrane as a carrier. The interim results of a clinical trial involving 700 patients with severe unilateral and bilateral LSCD revealed 70 and 50% success at

G.K. Vemuganti, M.D., DNB, FAMS, FICP (✉)
School of Medical Sciences, University of Hyderabad,
Prof C R Rao Road, Gachibowli, Hyderabad 500 046, Andhra Pradesh, India
e-mail: deanmd@uohyd.ernet.in; gvmd@uohyd.ernet.in

V.S. Sangwan, M.D.
Cornea and Anterior Segment, Ocular Immunology and Uveitis Services, Prof Brien Holden Eye Research Centre, L.V Prasad Eye Institute, Banjara Hills, Hyderabad 500 034, Andhra Pradesh, India

I. Mariappan, Ph.D.
Sudhakar and Sreekanth Ravi Stem Cell Biology Laboratory, Prof Brien Holden Eye Research Centre, L.V. Prasad Eye Institute, Banjara Hills, Hyderabad 500 034, Andhra Pradesh, India

D. Balasubramanian, Ph.D.
Prof Brien Holden Eye Research Centre, L.V Prasad Eye Institute,
Banjara Hills, Hyderabad 500 034, Andhra Pradesh, India

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_29,

the end of 3 and 5 years respectively. For patients affected by bilateral condition, options include use of allogenic tissues with immunosuppressive therapy or use of autologous alternative sources of epithelium like oral mucosal epithelium, both of which show limited success. The pre-requisites for cell therapy are that the desired cells should be grown in sufficient amounts, should survive, integrate and network with the host tissues and cause no harm to the recipient. All these criteria are fulfilled when limbal epithelial cell therapy is used for ocular surface reconstruction thus making it a successful model in the emerging field of regenerative medicine.

29.1 Introduction

The integrity of ocular surface is a cumulative function achieved by the co-ordination and interdependence of the ocular surface elements (epithelia and adnexa of the eye) and the tear film. The cornea, the central transparent part of the eye that contributes significantly to normal vision, is made up of five layers; the epithelium, Bowman's membrane, stroma, Descemet's membrane, and the endothelium. The homeostasis of epithelium is maintained by stem cells located in the region know as the limbus (Thoft and Friend 1983). In the normal uninjured state, LSC (limbal stem cells) are mitotically quiescent and maintained in a specialized limbal stromal microenvironment or "niche". However, on corneal epithelial wounding, stem cells located in the limbus (Hanna 1966; Davanger and Evensen 1971) proliferate to generate more stem cells and transient amplifying cells which then migrate centripetally so as to replace the damaged corneal epithelium. Any damage to this functional and physical interdependent structures leads to vision threatening conditions ranging from decreased to complete loss of vision posing a challenge to the clinicians to reconstruct the ocular surface. The most important of such conditions is the Limbal Stem Cell Deficiency (LSCD) caused by a number of individual or environmental or incident specific factors. (Dua and Azuara-Blanco 2000; Chen and Tseng 1991; Kruse et al. 1990). Limbal stem cell deficiency is a challenging clinical problem and the current treatment involves replenishing the depleted limbal stem cell (LSC) pool either by limbal tissue transplantation or by using cultivated limbal epithelial cells (LEC).

29.2 *Limbal Stem Cells*: Development, Stem Cell Function

Cornea is the transparent window on the ocular surface that allows light rays to pass through the anterior chamber and contributes to 60% of the total refractive power of an eye. Corneal surface consists of a 5–6 cell thickness stratified epithelial layer. The narrow zone of about 2 mm thickness between the cornea and the bulbar conjunctiva is known as the limbus and is widely accepted as the niche for the corneal epithelial stem cells (Fig. 29.1) (Hanna 1966; Srinivasan and Eakins 1979; Cotsarelis et al. 1989). The limbal epithelium is 10–12 cell layers thick, and

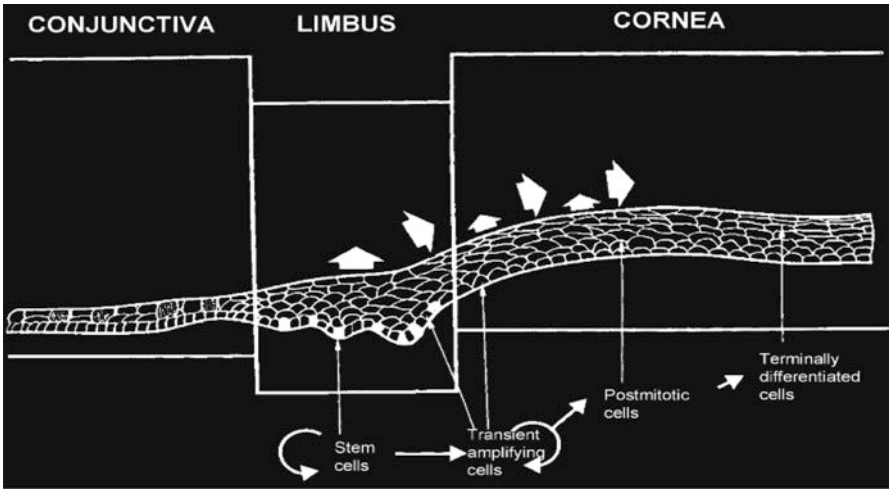


Fig. 29.1 Schematic diagram showing location of cornea, limbus and corneal epithelia. During homoestasis as well as during wound healign, the limbal stem cells from limbal region, proliferate, form transient amplying cells that move centripetally into the cornea, mature and differentiate into the corneal epithelial cells.

contains melanocytes, Langerhan cells and an underlying network of blood vessels in the limbal stroma. The limbal stroma with its overlying epithelium is arranged in radial fibrovascular elevations called the Palisades of Vogt, which alternate with epithelial rete ridges (Van Buskirk 1989). These palisades are present 360° all around the corneo-scleral rim but are most well defined in the inferior and the superior zones.

The stem cells located at the limbal region play an important role in the normal maintenance of the corneal surface. The corneal epithelium is a highly dynamic structure and is constantly renewed all through the lifetime of the eye. The activated stem cells from the limbus migrate centripetally to the central cornea and helps in tissue homeostasis. As explained by XYZ theory (Thoft and Friend 1983) of corneal maintenance, the combined rate of cell proliferation and centripetal migration of the activated limbal stem cells (XY component) is equal to the rate of cell loss by desquamation (Z component).

Limbal epithelial stem cells (LESC) share common features with other adult somatic stem cells including small size (Bickenbach 1981; Holbrook and Odland 1975) and high nuclear to cytoplasmic ratio (Epstein et al. 2005). They also lack expression of differentiation markers such as cytokeratins 3 and 12 (Schermer et al. 1986; Fatima et al. 2006). LESCs are slow cycling during homeostasis and therefore retain DNA labels for long time periods, however in the event of corneal injury they can become highly proliferative (Park et al. 2006; Cotsarelis et al. 1989; Haskjold et al. 1989; Thompson et al. 1991) and contribute actively to healing of the wound. These limbal stem cells divide asymmetrically to replenish and maintain the stem cell pool in the limbal niche. The limbal location of corneal epithelial stem cells

provides a number of functional advantages. The basal cells of the cornea are devoid of any pigments and are highly susceptible to radiation induced damages. But the limbal region is pigmented and therefore the basal stem cells do not face this constraint. Also, the limbal epithelium has a highly undulating epithelial- stromal junctions and therefore are resistant to the shearing forces and provides maximum protection to the corneal stem cells (Van Buskirk 1989). It is important to note that while the cornea is avascular, the limbal epithelium has a vascular bed which provides for the nourishment and maintenance of the limbal stem cells.

During wound healing process, the stem cells get activated and the transiently amplifying cells migrate in a centripetal manner which is a unique characteristic of the limbal epithelium. They undergo cell division and migrate centripetally towards the central cornea and also show a basal to upward movement towards the corneal surface. Once the epithelial cells leave the limbal basal layer or the limbal niche, they gradually activate the differentiation programme and differentiate into transiently amplifying cells (TAC) with lesser proliferative capacity and reduced stemness. Prior to or during the course of migration, these TAC become terminally differentiated and contribute to corneal wound healing by establishing cell-cell contacts through the formation of desmosomes, hemidesmosomes and tight junctions (Chen and Tseng 1990). Several reports in the past two decades have confirmed the limbal location of corneal epithelial stem cells and the role they play in regenerating the corneal epithelium (Sangwan and Tseng 2001; Zieske et al. 1992; Cotsarelis et al. 1989).

29.3 *Ocular Surface* Diseases- Regenerative Principles

The mechanism by which ocular surface health is ensured is built into the intimate relationship between ocular surface epithelium and the periocular tear film. Damage to the ocular surface caused by chemical, thermal, mechanical injuries, or immune-mediated diseases may results in limbal stem cell deficiency. LSCD is typically characterized by the invasion of conjunctival epithelium onto the corneal surface leading to conjunctivalization, neo-vascularization, subepithelial scarring and symblepharon formation resulting in corneal opacification, persistent or recurrent corneal epithelial defects and visual impairment apart from varying degrees of discomfort which includes redness, irritation, watering and light sensitivity in the affected eye (Vemuganti et al. 2009; Chen and Tseng 1990, 1991; Kruse et al. 1990). There are a variety of causes for the development of limbal stem cell deficiency (LSCD) Table 29.1. The definitive treatment for LSCD is limbal transplantation using auto or allografts of limbal tissues, each of which are associated with different risks and benefits. The success of limbal stem cell transplantation is determined by a variety of factors and may be adversely affected by concomitant lid pathology, dry eye and uncontrolled systemic disorders. Hence, the management of associated adenexal conditions such as eyelid/eyelash disposition and management of dry eye is a pre-requisite for a successful ocular surface reconstruction and needs to precede stem cell transplantation. Increasing knowledge in the biology of limbal stem cells

Table 29.1 Etiology of limbal stem cell deficiency (LSCD)

Primary LSCD
Hereditary aniridia
Ectodermal dysplasia or other congenital connective tissue disorders
Neurotrophic keratopathy
Sclerocornea
Secondary LSCD
Trauma
Chemical injuries
Acid injuries
Alkali injuries
Thermal injuries
Radiation injury
Ultraviolet radiation
Ionizing radiation
Systemic conditions
Steven Johnson Syndrome (SJS)
Ocular cicatricial pemphigoid (OCP)\Pseudopemphigoid
Multiple endocrine disorders
Vitamin A deficiency
Iatrogenic:
Multiple ocular surgeries, e.g.:-excision of pterygia, pseudopterygia, limbal neoplasm
Cyclocryotherapy
Antimetabolites (topical mitomycin C (MMC))
Systemic chemotherapy
Contact lens wear
Severe ocular surface diseases:
Keratoconjunctivitis sicca
Post-infectious keratitis
Neurotrophic keratitis
Vernal/atopic keratoconjunctivitis
Rosacea blepharoconjunctivitis
Phlyctenular disease
Tumors
Pterygium

have opened up new avenues of ocular surface regeneration using the principles of stem cells, regenerative medicine and tissue engineering (Sangwan and Tseng 2001; Tsubota et al. 1999; Sangwan et al. 2003).

29.4 Clinical Principles, Diagnostics, Indications of Ocular Surface Regenerative Therapies

The clinical principle in treating severe Limbal Stem Cell Deficiency is the replacement of depleted limbal stem cell stores by surgical intervention. Various surgical techniques of limbal transplantation using cadaveric (Tsubota et al. 1999)

or live-related donor tissues have evolved for the treatment of bilateral limbal stem cell deficiency (LSCD) (Rao et al. 1999; Daya and Ilari 2001). However, these methods have met with limited success as allografts require immunosuppression for indefinite period to avoid rejection problems (Solomon et al. 2002; Ilari and Daya 2002). Long-term systemic immunosuppression involves the risk of serious eye and systemic complications, apart from being a significant economic burden. For unilateral total or partial limbal stem cell deficiency (LSCD) direct or cultivated autologous limbal transplantation works very well. With direct limbal stem cell transplantation there is a need for transplanting 3–6 o' clock hours of healthy limbus from the contralateral eye. This technique could potentially lead to limbal stem cell deficiency at the donor site. To overcome this, a novel way of expanding the stem cell population of limbal tissue has been adopted and has revolutionized the field of ocular surface reconstruction in the last decade.

In case of unilateral LSCD, the treatment option includes transplantation of (i) human amniotic membrane (hAM) (Tseng et al. 1998) (ii) healthy limbal tissues or (ii) in vitro cultured limbal epithelial stem cells. Over the past decade, cultured limbal epithelial stem cell transplantation (CLET) has been widely accepted as a standard approach for the ocular surface reconstruction of patients with LSCD (Pellegrini et al. 1997; Schwab et al. 2000; Tsai et al. 2000a, b; Nakamura et al. 2003; Sangwan et al. 2005; Baradaran-Rafii et al. 2010; Kolli et al. 2010). Cultured limbal epithelial transplantation (CLET) followed by penetrating keratoplasty (PKP) has resulted in long term graft survival and improved visual outcome (Sangwan et al. 2005) (Fig. 29.2). Another technique described for focal LSCD is ipsilateral translocation of healthy limbal tissue to an area of partial LSCD. In a small series of patients, this has been shown to provide good outcomes (Nishiwaki-Dantas et al. 2001). However this approach is not well studied and understood for the management of partial LSCD. The visual acuity of a patient with ocular surface disease who has undergone limbal stem cell transplantation may improve without any further surgical intervention. However, in cases where the corneal stromal opacification hampers visual recovery, a penetrating keratoplasty (PK) may be necessary. While some authors suggest PK and limbal stem cell transplantation to be done at one sitting (Rao et al. 1999; Theng and Tan 1997), Croasdale et al. (1999) recommends an interval of 3 months between the two and some others (Ilari and Daya 2002) recommend a deep lamellar keratoplasty 1 year post limbal stem cell transplantation, if the endothelium is healthy. In our experience, the latter approach of staged ocular surface reconstruction followed by subsequent corneal transplantation (lamellar in any situation where the endothelium is deemed healthy and penetrating in eyes with endothelial injury) is the preferred approach for visual rehabilitation of such eyes.

In case of bilateral LSCD, allografting is done using limbal tissue from a live related donor or from cadaveric sources. However this involves the associated risk of graft rejection and therefore requires a long term administration of immune suppressive drugs which becomes unaffordable for low income groups resulting in non-compliance, graft rejection and are predisposed to opportunistic microbial infections as well. Therefore there exists a need for an alternative autologous tissue source that

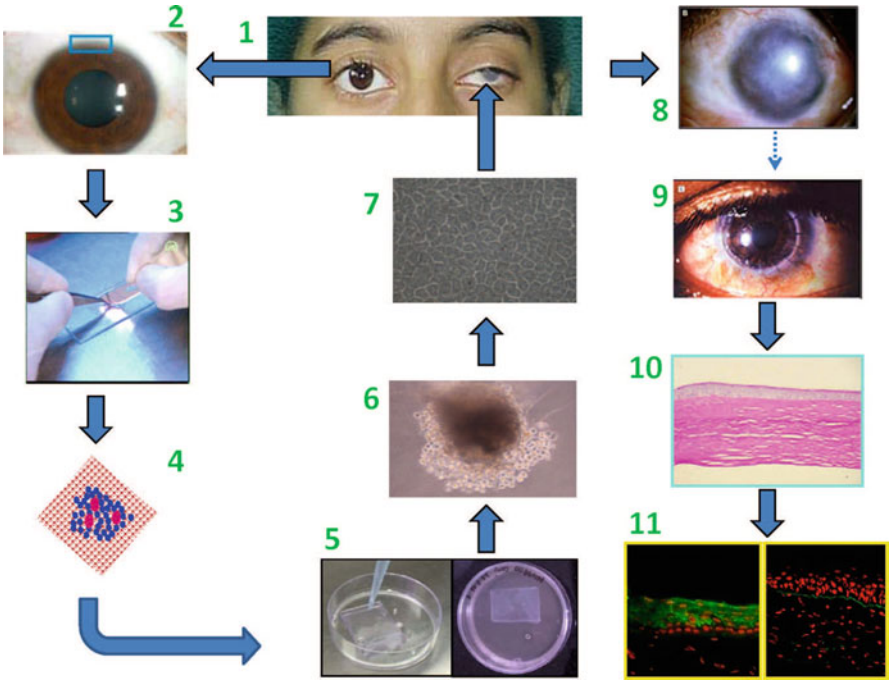


Fig. 29.2 The figure shows the chronological events for cultivated limbal epithelial transplantation. (*1*) Identifying patients with clinical features of unilateral severe limbal stem cell deficiency. (*2*) Harvest limbal biopsy from the healthy contralateral eye for ex-vivo expansion. (*3*) The harvested biopsy is fragmented into tiny explants for cultivation. (*4*) Ex-vivo expansion of cells: By a feeder cell free method of explant culture on denuded human amniotic membrane. (*5*) The cultures are incubated at 37°C with 5% CO2 and cultured for 2 weeks using human corneal epithelial medium. (*6*) The cultures are observed for growth of cells. Note the presence of cells arising from the edge of the explant by day 2–3. (*7*) Formation of a monolayer of epithelial cells by day 10–12. (*8*) Reconstruction of the epithelium of the diseased eye – By transplanting the epithelial sheet derived from autologus limbal tissue on hAM. (*9*) After stablization of the ocular surface by the transplanted cells, some of the patients may undergo penetrating keratoplasty for visual rehabilitation. (*10*) The study of the corneal button removed from the patient after cultivated limbal epithelial transplantation provides the objective proof of survival, maturation and integration of transplanted cells. (*11*) The corneal epithelim is intact, stratified and adherent to the corneal stroma. Note the formation of basement membrane as seen by immunofluorescence studies for Collagen IV- a basement membrane marker

can functionally replace the corneal epithelium. In order to address these issues, a few groups have attempted to use autologous conjunctival or oral mucosal epithelial cells as an alternative to limbal epithelium for corneal surface reconstruction. Cultivated oral mucosal epithelial transplantation (COMET) has been successfully used to treat patients with severe bilateral corneal defects with good clinical outcome in terms of ocular surface stabilization and marginal improvement in terms of visual acuity (Ang et al. 2006; Hayashida et al. 2005; Nishida et al. 2004). COMET followed optical PK has been reported by one group to achieve good improvement in the visual outcome with a stable corneal graft (Ma et al. 2009).

The technique of ex vivo expansion of limbal stem cells and their subsequent transplantation offers certain advantages. A small amount of tissue needs to be harvested – either from the healthy fellow eye of a patient with unilateral LSCD (contra lateral) or from the healthy limbus of a partially affected eye (ipsilateral) or from the other phthisical eye with a healthy limbus or from the healthy eye of the patient's living related donor or from the cadaveric limbal rings in case of cultivated limbal allograft. This reduces the risk of an iatrogenic LSCD in the donor eye and allows the possibility of a second biopsy being taken, if required. Owing to the absence of the antigen-presenting Langerhans' cells, these bioengineered sheets are less immunogenic. In vitro culture also ensures that cells are viable and proliferating as opposed to the cadaveric limbal transplantation. We have shown in our laboratory that (Vemuganti et al. 2004) chances of cell growth from live limbal tissues is 100% while those from the cadaveric tisues showed only 51.6% cell growth.

LSCD may occur due to primary or secondary causes (Puangsricharern and Tseng 1995). Primary LSCD is characterized by the absence of identifiable external factors. In these cases, the dysfunction of stromal microenvironment of limbal epithelial stem cells results in gradual loss of stem cell population or generation and amplification of transient amplifying cells (Vemuganti et al. 2009). Secondary LSCD occurs due to the destruction of LSCs by external factors as seen in trauma, systemic conditions, iatrogenic causes and severe ocular disease (Vemuganti et al. 2009).

We have earlier reported our surgical techniques for cultivated limbal epithelium transplantation (Sangwan et al. 2005). After obtaining informed consent from the patients or guardians, limbal biopsy is obtained from the healthy contralateral eye or from the healthy area of the same eye for autologus procedures. The procedure involves careful dissection of a 2×2 mm piece of limbal epithelium with 0.5 mm into clear corneal stromal tissue at the limbus under strict aseptic conditions using local or general anesthesia, depending on the patient's preference and age. The limbal tissue that contains limbal epithelial cells at the pigmented line (palisades of Vogt) and a part of the corneal stroma is excised. If limbal stem cell deficiency is bilateral, allografting is done using limbal tissues harvested from the live-related donor or from cadaveric limbal rings obtained from the eye bank. Earlier reports from our group (Vemuganti et al. 2004) have shown that fresh limbal tissue with 100% viability is preferable to the preserved cadaveric limbal tissues with 51.6% viability for culture.

However the technique of culturing these cells has been varying with different investigators. The variations in the culture technique involves the preference for the type of biopsy (cadaveric or fresh), choice of substrate (human amniotic membrane or Fibrin or Collagen), presence or absence of feeder cells, type of culture medium (HEC or DMEM), use of culture inserts, use of autologous human serum or fetal calf serum, submerged or air-lift culture technique (Vemuganti et al. 2009). Based on our experience from treating more than 700 cases, we believe that a feeder-cell free method of submerged explant culture technique on human amniotic membrane using the autologus serum is a cost effective and efficient method of generating corneal epithelial monolayer for clinical transplantation.

29.5 Standardized Treatment Technologies

An ideal substrate for cultivation of limbal epithelial cells should have high optical clarity, appropriate refractive index, dimensions as that of cornea, toughness to withstand surgical procedure i.e., adequately robust for implantation, non-toxic, non-immunogenic, non-inflammatory and most importantly should promote corneal epithelial cell proliferation and innervation. Human amniotic membrane has been extensively used for ocular surface reconstruction and has the required properties to facilitate epithelial cell growth, suppresses inflammation, and also possesses the anti-bacterial and anti-apoptotic properties, thus making it an ideal scaffold for clinical application.

We used the standard protocol proposed by Kim and Tseng (1995) to prepare the human amniotic membrane. In brief, the placenta (which has two layers called the amnion and chorion) obtained from the caesarian section delivery was used to prepare human amniotic membrane (hAM). After screening the donor for HIV, HBs Ag and VDRL, the placenta was placed in a sterile pan and washed repeatedly with antibiotic containing ringer lactate/normal saline until water runs clear. The placenta was then transferred aseptically to another sterile pan and carried to the laminar flow hood, which was pre-cleaned, and UV sterilized. AM was peeled, separating the amnion and chorion. The stretched membrane was cleaned using a cotton swab and intermittent wetting with ringer lactate/normal saline using wash bottle. Once a clean transparent approximately 2×2 cm area (7.5×7.5) became available, the nitrocellulose paper was attached on the chorion side keeping the epithelium side up. The hAM was cut around the paper while rolling the edges on the other side of the paper. (hAM should stick to the nitrocellulose paper perfectly without gaps or air-bubbles). The nitrocellulose paper was then cut to get small pieces of membrane as per requirement. The hAM pieces (2.5×2.5, 2.5×5, 5×5 cm) were then placed in vials containing Dulbecco's Modified Eagles Medium (DMEM) and 50% glycerol and stored at −70°C. Just before use, the hAM was thawed at 37°C for 30 min. The composition and instructions to prepare the Human Corneal Epithelial (HCE) medium used to culture these cells is described in Table 29.2.

A careful clinical examination is required for all the potential patients of cultivated limbal epithelial transplantation (CLET). Ocular adnexa should be examined for any defects, if found to have lid notch or defects, then it should be repaired prior to CLET. The patients should not have surface anesthesia as found in patients with herpes zoster ophthalmicus or due to seventh nerve paralysis. If there is underlying immune-mediated inflammation as seen in ocular cicatricial pemphigoid (OCP) or Stevens-Johnson syndrome (SJS), then it should be treated with appropriate therapy before doing CLET. If the limbal stem cell deficiency (LSCD) is due to chemical injury then enough time should be allowed before attempting ocular surface reconstruction so that inflammation subsides and chances of survival of transplanted epithelial cells improves. The visual potential of the eye should be carefully assessed before doing the surgery especially in children where there is a possibility of amblyopia.

Table 29.2 Composition of Human Corneal Epithelial Medium (HCEM) for culture of limbal epithelial cells

S. No.	Ingredients	Quantity per liter	Company
1	Minimal Essential Medium	5.05 g	Sigma, Cat. No. M0644
2	Nutrient Mixture Ham's F-12	5.30 g	Sigma, Cat. No. N6760
3	Sodium bicarbonate	1.688 g	Sigma, Cat. No. S5761
4	Penicillin G sodium salt (100 U/ml)	0.1 MU	Sigma, Cat No. P3032
5	Stereptomycin sulphate (100 μg/ml)	100 mg	Sigma, Cat No. S9137
6	Gentamicin sulfate (5 μg/ml)	5 mg	Sigma, Cat No. G1264
7	Amphoterecin B (2.5 μg/ml)	10 ml of 100X stock	Sigma, Cat No. A2942
6	Epidermal Growth Factor (10 ng/ml)	100 μl of 100 μg/ml stock	Sigma, Cat No. E9644
7	Insulin (5 μg/ml)	100 μl of 50 mg/ml stock	Sigma, Cat No. I0259

Method of Preparation: Add the first two ingredients in 500 ml of Milli Q water, in a sterile 1 l flask/beaker, add sodium bicarbonate, add the antibiotics and stir to dissolve. Adjust the pH to 7.2 with 1 N HCl or 1 N NaOH while stirring and make up the volume to 900 ml. Add the growth factors, mix well and filter sterilize the medium by vacuum filtration through a 0.22 μm membrane filter. Perform sterility check by streaking on to chocolate agar and blood agar plates and by inoculating into thioglycollate broth before use. The medium is then stored at 4°C. The shelf life of the medium is about 15 days as after that the pH of the medium increases as indicated by change in color (phenol red) towards more pinkish

Separation of autologous serum: About 10-ml blood is drawn from the patient in non-heparin, 15 ml graduated falcon tubes. The blood is kept at room temperature undisturbed for a couple of hours to allow the settlement of cellular components. It is then centrifuged at 2,000 rpm for 10 min. The serum supernatant is pipetted out in a fresh falcon and filter sterilized using 0.22 μm millipore syringe filters, and used in the culture medium at 10% concentration

The surgery is performed under local or general anesthesia, based on patient's choice as well as patient's age. In younger children general anesthesia is preferred. A drop of epinephrine (1: 1,000) is instilled in the conjunctival cul-de-sac prior to pannus excision to decrease bleeding during the procedure. Dissection of ocular surface pannus is started 2–3 mm behind the limbus if this landmark is still visible. Blunt tip spring scissors or conjunctival scissors are used to initiate the dissection by lifting the pannus and dissection is continued all around the limbus with a # 15 blade on Bard Parker handle. A combination of sharp and blunt dissection from the periphery towards the central area is carried out to clean the cornea. It is important to pay attention to the thickness of the corneal tissue, which may be variable due to the effects of chemical or disease process, and it is vital not to perforate the cornea. Any accidental perforation, which can be identified by softening of the globe, should be repaired either by suturing or by application of fibrin tissue sealant. If the perforation is too large to be repaired then lamellar or penetrating keratoplasty must be performed along with cultivated limbal stem cell transplantation. Perforation is uncommon if the tissue planes are respected by the surgeon. The ocular surface is

then cleared of the fibrovascular pannus and hemostasis is achieved by judicious use of bipolar cautery. The adhesions between the eyeball and lids (symblephara) are released taking due care not to injure the extraocular muscles and other tissues. Fornix is reconstructed using amniotic membrane, and fornix-forming suture if indicated. At this stage the corneal thickness is assessed clinically and by pachymetry. If the recipient corneal thickness is less than 300 μm, then a lamellar corneal grafting is done to restore the thickness of the cornea; this will help in supporting the epithelization of ocular surface following cultivated epithelial transplantation. At this stage, the cultivated limbal epithelium on human amniotic membrane is brought on to the ocular surface, cell side up, aseptically, and the membrane containing the cultured cells is gently spread over the cornea as well as limbus without damaging or dislodging the cells. The area of maximum number of visible explants with surrounding cells is kept at the center of cornea and the peripheral part of membrane is trimmed using blunt tipped scissors. There are two ways of anchoring the composite of membrane and cells on to the ocular surface. If sutures are used then, 10-0 monofilament nylon material is preferred. The tissue should be anchored using circumferential, interrupted sutures at the limbus. Peripheral edge of the membrane is sutured to conjunctiva with 8-0 polyglactin (Vicryl™) sutures. Usually 6–8 interrupted sutures are enough to secure the membrane and care should be taken to avoid any folds or wrinkles of the membrane. If they are present, then the tissue should be stretched and unfolded, holding the peripheral part of the membrane. The central part of the membrane, which has maximum cellular growth, is never touched because it is critical for re-epithelization. If there is inadvertent needle perforation, recognized by softening of the eye, then the suture should be taken at another location. Needle perforations usually do not require repair. If sutureless surgery is planned, the fibrin tissue sealant (Tissel™ Kit; Baxter AG, Vienna, Austria) is put on the ocular surface using a tuberculin syringe with 27 gauge needle. Both components are put one after another and the membrane with grown cells is brought on the ocular surface and spread as explained above. Excess membrane is trimmed and peripheral part of the membrane is ironed out in order to avoid wrinkles or folds of the membrane. The membrane is touched with a surgical sponge in order to test if the adhesion is sufficient to hold the tissue in place. Sometime there may fluid accumulation under the membrane during postoperative period. To avoid this complication two small holes are made in the membrane at limbus at 5 o' clock and 7 o' clock so the fluid is drained out.

At the conclusion of the surgery, one drop of 2.5% povidone iodine is instilled; use of a bandage contact lens is optional. Some surgeons recommend using a bandage contact lens for the first few weeks, to avoid loss of cells due to blinking. For the same reason some surgeons prefer to induce ptosis by injecting botulinum toxin in orbicularis oculi. We do not use any of these techniques because we believe that there are firm adhesions between the membrane and growing cells. The technique described above is for total limbal stem cell deficiency where fibrovascular pannus covers the entire ocular surface. If there is focal or sectoral deficiency the surgical technique is tailored to cover the defect created by removal of abnormal tissue. Either sutures or fibrin glue can be used to anchor the membrane with cultivated

cells to the ocular surface. Briefly, the procedure for the transplantation of cultivated limbal epithelial cells is represented in Fig. 29.2.

Following the cultivated limbal epithelium transplantation, all the patients are treated with prednisolone acetate 1% eye drops or any other equivalent topical steroids drops, eight times a day, tapered to once a day in 5–6 weeks, and ciprofloxacin hydrochloride 0.3% eye drops, four times a day, for 1 week. Ciprofloxacin hydrochloride 0.3% eye drops are continued if an epithelial defect is present or as long as the bandage contact lens is used. If a limbal allograft is done then the patient should be given systemic immunosuppression, as has been reported earlier (Sangwan et al. 2005). The patients are examined on postoperative day one, 1 week, 2 weeks, 5 weeks and monthly thereafter for 6 months. Thereafter, follow up is customized to success or failure of the procedure. If successful and does not require further interventions, then follow up is every 6 month for 3 years and thereafter annually. For failed surgeries process could be repeated or appropriate alternative therapy offered. Each examination includes a complete history, noting of new ocular or systemic symptoms, and complete evaluation of recipient as well as donor sites and any signs of neovascularization or surface instability. Sutures are removed when indicated (loose or vascularized). Epithelialized sutures are left indefinitely. Some of the patients with CLET may require penetrating keratoplasty or deep anterior lamellar keratoplasty if the vision does not improve with CLET alone.

Ocular surface reconstruction in limbal stem cell deficiency is a multi-staged procedure, with the patient often requiring several surgical interventions for visual rehabilitation. Some of these patients may require cataract extraction after successful CLET which can be done in a standard way. If there is stromal scarring and CLET has been successful then penetrating keratoplasty (PKP) or deep anterior lamellar keratoplasty (DALK) can be done to improve the vision.

Sangwan et al. (2005) described the early results of penetrating keratoplasty (PKP) in patients who had previously undergone cultivated limbal epithelium transplantation. Demographics, primary etiology, type of limbal transplantation, ocular surface stability, visual acuity, graft clarity, and complications were reviewed. Histopathologic features of the recipient corneal buttons were studied with special attention to epithelial status (Fig. 29.2). PKP was performed at a mean interval of 7 months (range, 2–12 months) following cultivated limbal epithelium transplantation (autologous, n = 11; allogenic, n = 4). Fourteen (93%) of the 15 eyes had a successful corneal graft with a stable corneal epithelium. Preoperative best-corrected visual acuity was less than 20/200 in 14 of the 15 eyes. At a mean follow- up of 8.3 months after PKP, the best-corrected visual acuity was more than 20/60 in 8 eyes, 20/200 to 20/60 in 5 eyes, and less than 20/200 in 2 eyes. Three of the 15 eyes experienced corneal allograft rejection, which was managed successfully. None of the limbal epithelial allografts showed signs of rejection.

These cases did not show a high rejection rate (overall rejection rate, 20%) despite the young age of the recipients and stromal vascularization or a high non–rejection-related failure as expected in cases of chemical burns. This could be attributed to the cultivated limbal epithelium transplantation procedure preceding the PKP, which continued to supply healthy epithelium after PKP. Similarly, the fewer corneal graft rejection episodes could be due to the stepwise, versus the simultaneous,

approach, which included ocular surface reconstruction by cultivated limbal epithelium transplantation in the first step and PKP in the second.

The proponents of simultaneous limbal stem cell transplantation and penetrating keratoplasty highlight the potential of this approach to minimize recipient antigenic load by using the same donor tissue for both the limbal stem cells and the keratoplasty procedure. However, ex vivo expanded limbal epithelium transplantation requires a minimum period of 2 weeks for the expansion of limbal stem cells. A staged approach allows achievement of a stable ocular surface prior to the penetrating keratoplasty and allows sufficient time for inflammation to subside, thereby increasing the chances of a successful penetrating keratoplasty. The low rejection rate may also be ascribed to the fact that the cultivated limbal epithelium is devoid of Langerhans cells (Holland et al. 1987) and the anti-inflammatory role of the amniotic membrane carrier.

Since the patients have already undergone pannus resection with or without superficial keratectomy, the recipient corneal stromal bed is usually thin and irregular, which could result in postoperative astigmatism. In addition, associated conditions, such as eyelid abnormalities, glaucoma, and dry eye syndrome, may affect the outcome and hence must be treated before PKP.

29.6 Clinical Studies, Experience, Outcome/Side Effects of "Ocular Surface" Regenerative Therapies

While the literature has consistently seen reports on the short and intermediate term efficacy of stem cell transplantation for ocular surface reconstruction, it must be mentioned that all of these studies are limited in that they are retrospective, non-randomized, the number of eyes in the cohorts being small and relatively short durations of follow-up. It is clear from the literature that autograft transplantation is efficacious for both transplanted limbal tissue (Kenyon and Tseng 1989; Rao et al. 1999; Shimazaki et al. 2006) as well as for cultivated limbal epithelial transplants (Tsai et al. 2000a, b; Rama et al. 2001; Sangwan et al. 2005, 2006), but is limited by relatively short follow-ups.

Ocular surface reconstruction by cell therapy provides a unique opportunity to document the survival, networking, and integration of transplanted cells, through various techniques. A thorough clinical evaluation for presence of epithelial integrity, lack of staining by fluorescein dyes, absence of vascularization, and corneal clarity is indicative of successful transplantation. A novel form of non-invasive method of documenting the survival and stratification of the transplanted cells is by clinical confocal microscopy which documents the multilayering of corneal epithelial cells, and remnants of the degraded HAM (if any). A minimally invasive method of documenting the proof of survival of transplanted cells is by impression cytology of the ocular surface which provides information on the phenotype of the cells lining epithelium in the central corneal region. Presence of corneal phenotype (K3+, K19−) and the absence of goblet cells indicate suggest successful transplantation.

A recent study by Pauklin et al. (2009) on pannus tissue excised from LSCD patients showed that the epithelial lineage marker expression (Keratins- K3, K12, K19 and MUC5AC) was seen only in the areas close to the conjunctiva instead of in the central cornea of LSCD cases, but were reverted to the normal central corneal phenotype with significant reduction in inflammatory markers (IL-1alpha, IL-1beta, ICAM-1, VCAM-1 and VEGEF) post-LSCT.

It is important to note that the transplantation of limbal tissue or cells is done to restore the surface epithelium and corneal clarity and visual acuity needs to be assessed and evaluated in due course of time. Patients who show surface stabilization but lack corneal clarity may require corneal transplantation for visual rehabilitation. The corneal specimen received from patients undergoing PK following CLET for visual rehabilitation provide us the unique opportunity to objectively document the proof of surviving cells through histological studies.

Earlier results of PKP in these eyes was not very encouraging probably because of the limited life span and limited proliferative potential of the TACs from PKP specimen, was insufficient to restore the ocular surface epithelium on a long-term basis. Our data (unpublished) showed the presence of stratified corneal epithelium and the establishment of basement membrane (collagen IV staining) in about 75% of cases (Fig. 29.2). This proves the concept that the transplanted limbal stem cells repopulate the damaged host corneal tissue and becomes functional as evident by the formation of its own basement membrane and by the expression of cornea specific markers.

If resources and facilities are available, the corneal epithelial cells from the PK tissues can be harvested to provide a molecular proof of cell survival through DNA finger printing in allogenic CLET treated patients. Studies have shown that the surviving donor limbal epithelial cells in allogenic transplantations are responsible for the long-term graft survival. The clinical efficacy of limbal transplantation does not necessarily correlate with the survival of donor cells on the ocular surface. Some reports have shown the presence of a chimera, wherein the surviving corneal epithelium constitutes both the donor and the recipient cells, probably indicating that the surviving donor cells have participated in tissue repair. The use of laser capture microdissection (Zhou et al. 2006) and corneal impression cytology (Nelson 1988; Tseng 1985; Williams et al. 1995) allows for a clean examination without involving the stroma or other contaminants.

Williams et al. (1995) have investigated the survival of donor-derived epithelial cells based on impression cytology after limbal stem cell transplantation (allograft), using short tandem-repeat DNA polymorphisms (microsatellites) to distinguish the donor and recipient cells and showed that cells of donor genotype were present over the grafted areas at the time of surgery but were not detected in the central cornea until 12 weeks postoperatively, indicating that repopulation of the epithelial surface from the transplanted limbal stem cells took considerable time while only the recipient-type cells were detected in the grafted eye by 20th week. Henderson et al. (1997) attempted to investigate donor cell survival following corneal limbal stem cell grafting using amelogenin gene probe (a Y-specific DNA probe) with PCR. The same group later (Henderson et al. 2001) demonstrated that a combination of impression cytology and single cell DNA fingerprinting is suitable for detecting transplanted cells after corneal limbal allografting.

Although there are no known cases of limbal dysfunction after removal of donor tissue from a healthy eye, the effect of initial trauma of healthy eye cannot be ruled out. Irreversible damage caused by the removal of limbal tissue from a partially stem cell deficient eye may be reduced by cultivated limbal epithelial sheets limiting the amount of excised tissue.

29.7 Outcome of Cultivated Limbal Epithelium Transplantation

Clinical outcome is determined by noting the improvement in ocular surface stability, as judged by non-recurrence of conjunctivalization and the absence of epithelial defects. There are several case reports and small case series reported in the literature using this technique. Table 29.3 summarizes the clinical outcome of CLET. We have reported the largest series of autologous cultivated limbal epithelial transplantation (CLET) in 2006 (Sangwan et al. 2006). Eighty-eight eyes of 86 patients underwent autologous CLET between 2001 and 2003, alkali burn was the most common cause of LSCD (n = 56), and 61 of these 88 eyes had total LSCD. Success was achieved in 73.1% (95% CI 63.3–82.9) and BCVA on Snellen's chart improved from 17/78 (21.8%) eyes to 41/78 (52.6%) having functional ambulatory vision (>20/200) postoperatively. We have reported early results of penetrating keratoplasty (PKP) following CLET. 15 of 125 patients underwent PKP following CLET at a mean interval of 7 months. Fourteen of the 15 eyes had a successful corneal graft with stable corneal epithelium (Sangwan et al. 2005).

Koizumi et al. (2001) reported transplantation of allogenic cultivated corneal limbal epithelium from donor corneas. The cells were cultivated on denuded amniotic membrane with 3 T3 fibroblast feeder cell layer. Air-lifting technique was used to achieve stratification of the epithelium. Thirteen eyes with total LSCD from 11 patients were selected for the procedure. The cultivated epithelium was transplanted to the diseased eye. Simultaneous lamellar keratoplasty was performed in 5 patients with associated stromal scarring. All the recipients were started on an immunosuppression regime. After 48 h all of the 13 eyes showed regained epithelial integrity. On follow-up for 6 months, 10 of the 13 eyes (77%) showed improved BCVA, a gain of at least two lines in the Snellen's chart. However, 3 eyes experienced epithelial rejection. This finding underscores the importance of proper immunosuppression for the allograft technique.

29.8 Conclusions and Future Perspectives on "Ocular Surface" Regenerative Therapies

Though severe limbal stem cell deficiency is a rare disease, it has a unique role in unraveling the mysteries of stem cell concept in the limbus, and has set precedence in the field of cell therapy. This also has the unique distinction of bringing together the clinicians, scientists and cell biologists together in taking the science from bench

Table 29.3 Clinical outcome of CLET

Author/year	Type of LSCD	Intervention	No of eyes	Mean follow up (months)	Overall success rate	Improved BCVA from baseline no./total (%)	Comments
1. Pellegrini et al. (1997)	Total – 2	Autologus LSCT	2	24	Stable ocular surface	Improvement in BCVA	
2. Schwab et al. (2000)	Total – 7 Partial – 7	Autologus LSCT – 10 Allograft LSCT – 4	14	13	Stable ocular surface in 6/10 of autografts and all 4 of allografts	All patients improved in BCVA. >20/200 in 12/14 patients And>20/30 in 7/14 patients	
3. Tsai et al. (2000a, b)	Total	Autografts	6	15	All eyes had stable ocular surface	83% BCVA improved from 20/112 to 20/45	
4. Rama et al. (2001)	Unilateral severe	Autologous LSCT	18	18.6		7/18 (38.9%)	
5. Koizumi et al. (2001)	Total	Allografts – 13	13	11.2	All eyes had stable ocular surface	BCVA improved by 2 lines or more in 10/13 eyes.	Subconjunctival tissue treatment with 0.04% mitomycin C
6. Shimazaki et al. (2002)	Total – 13	Allograft LSCT – 13	13	NA	46.2% 3/8 eyes developed partial conjunctival invasion 2 eyes later developed epithelial defects		Corneal perforation-4 eyes Infectious keratitis-2 eyes
7. Sangwan et al. (2005)	Total – 14, partial – 1	Autologous LSCT – 11 Living related allograft LSCT – 3 nonrelated allograft LSCT – 1	15	15.3		10/15 (67%)	In 15 cases PKP was done later

8.	Sangwan et al. (2006)	Bilateral – 4 Unilateral – 84	Autologus LSCT	86	18.3	Improvement in ocular surface –70%	BCVA improvement –40%	
9.	Daya et al. (2000)	Total – 10	Allografts – 10	10	28			
10.	Nakamura et al. (2006)	Total – 9	Allograft LSCT – 7 Autologus LSCT – 2	9	14.6	Stable ocular surface at last follow up	Improvement in more than two lines	
11.	Kawashima et al. (2007)	Total – 6	Autologus – 2 Allografts non related – 3 Allograft related – 1	6	25	Stable ocular surface at last follow up	Improvement in BCVA in 4 out if 6 eyes	All eyes subsequently underwent keratoplasty for visual rehabilitation
12.	Shortt et al. (2008)		Autologus – 3 Allografts – 7	10	13	60% (autografts 33%, allografts 71%)	Improvement in more than 2 lines of preoperative BCVA	
13.	Baradaran-Rafii et al. (2010)	Total unilateral – 8	Autologus – 8 Subsequently PKP – 4	8	34.0	7 eyes – stable corneal epithelium with decrease in opacification and vascularization	Progressive sectorial conjunctivalization in all cases with subsequent PKP	
14.	Kolli et al. (2010)	Unilateral Total – 8	Autologus		19 months	100% stable ocular surface epithelium	BCVA improved in 5 eyes, remained same in 3	

to bedside, and from protocol to practice. Thus ocular surface reconstruction has served as a model system which fulfilled the principles of successful cell therapy wherein the desirable cell source is available in adequate quantities, well characterized and the surviving transplanted cells were documented to survive, network, integrate and restore the function of the target tissue. Though the clinical outcome is definitely rewarding in most experienced hands, a few aspects that require further attention include objective quantification of transplanted cells, development of tissue and cell banking facilities, and a non-invasive method of in-vivo cell tracking so as to understand the homing and survival of transplanted cells into the limbal niche.

Acknowledgements The authors wish to acknowledge all the patients of limbal stem cell deficiency who participated in the study. We wish to profusely thank Dr. G Chandra Sekhar, Director, LV Prasad Eye Institute, Dr. Gullapalli N Rao, Chairman, LV Prasad Eye Institute for their constant support and guidance. We also thank Karthik for assisting in figure designing.

References

Ang LP, Nakamura T et al (2006) Autologous serum-derived cultivated oral epithelial transplants for severe ocular surface disease. Arch Ophthalmol 124:1543–1551

Baradaran-Rafii A, Ebrahimi M et al (2010) Midterm outcomes of autologous cultivated limbal stem cell transplantation with or without penetrating keratoplasty. Cornea 29(5):502–509, Epub ahead of print

Bickenbach JR (1981) Identification and behavior of label-retaining cells in oral mucosa and skin. J Dent Res 60:1611–1620

Chen JJ, Tseng SC (1990) Corneal wound healing in partial limbal deficiency. Invest Ophthalmol Vis Sci 31:1301–1314

Chen JJ, Tseng SC (1991) Abnormal corneal epithelial wound healing in partialthickness removal of limbal epithelium. Invest Ophthalmol Vis Sci 32:2219–2233

Cotsarelis G, Cheng SZ et al (1989) Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell 57:201–209

Croasdale CR, Schwartz GS et al (1999) Keratolimbal allograft: recommendations for tissue procurement and preparation by eye banks, and standard surgical technique. Cornea 18:52–58

Davanger M, Evensen A (1971) Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature 229:560–561

Daya SM, Ilari FA (2001) Living related conjunctival limbal allograft for the treatment of stem cell deficiency. Ophthalmology 108:126–133; discussion 133–134

Daya SM, Bell RW et al (2000) Clinical and pathologic findings in human keratolimbal allograft rejection. Cornea 19:443–450

Dua HS, Azuara-Blanco A (2000) Limbal stem cells of the corneal epithelium. Surv Ophthalmol 44:415–425

Epstein SP, Wolosin JM et al (2005) P63 expression levels in side population and low light scattering ocular surface epithelial cells. Trans Am Ophthalmol Soc 103:187–199; discussion 199

Fatima A, Sangwan VS et al (2006) Technique of cultivating limbal derived corneal epithelium on human amniotic membrane for clinical transplantation. J Postgrad Med 52:257–261

Hanna C (1966) Proliferation and migration of epithelial cells during corneal wound repair in the rabbit and the rat. Am J Ophthalmol 61:55–63

Håskjold E, Bjerknes R et al (1989) Cell kinetics during healing of corneal epithelial wounds. Acta Ophthalmol (Copenh) 67:174–180

Hayashida Y, Nishida K et al (2005) Ocular surface reconstruction using autologous rabbit oral mucosal epithelial sheets fabricated ex vivo on a temperature responsive culture surface. Invest Ophthalmol Vis Sci 46:1632–1639
Henderson TR, McCall SH et al (1997) Do transplanted corneal limbal stem cells survive in vivo long-term? Possible techniques to detect donor cell survival by polymerase chain reaction with the amelogenin gene and Y-specific probes. Eye 11:779–785
Henderson TR, Findlay I et al (2001) Identifying the origin of single corneal cells by DNA fingerprinting: part II – application to limbal allografting. Cornea 20:404–407
Holbrook KA, Odland GF (1975) The fine structure of developing human epidermis: light, scanning, and transmission electron microscopy of the periderm. J Invest Dermatol 65:16–38
Holland EJ, DeRuyter DN et al (1987) Langerhans cells in organ-cultured corneas. Arch Ophthalmol 105:542–545
Ilari L, Daya SM (2002) Long-term outcomes of keratolimbal allograft for the treatment of severe ocular surface disorders. Ophthalmology 109:1278–1284
Kawashima M, Kawakita T et al (2007) Phenotypic study after cultivated limbal epithelial transplantation for limbal stem cell deficiency. Arch Ophthalmol 125:1337–1344
Kenyon KR, Tseng SC (1989) Limbal autograft transplantation for ocular surface disorders. Ophthalmology 96:709–722; discussion 722–723
Kim JC, Tseng SC (1995) Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea 14:473–484
Koizumi N, Inatomi T et al (2001) Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. Ophthalmology 108:1569–1574
Kolli S, Ahmad S et al (2010) Successful clinical implementation of corneal epithelial stem cell therapy for treatment of unilateral limbal stem cell deficiency. Stem Cells 28:597–610
Kruse FE, Chen JJ et al (1990) Conjunctival transdifferentiation is due to the incomplete removal of limbal basal epithelium. Invest Ophthalmol Vis Sci 31:1903–1913
Ma DH, Kuo MT et al (2009) Transplantation of cultivated oral mucosal epithelial cells for severe corneal burn. Eye (Lond) 23:1442–1450
Nakamura T, Koizumi N, Tsuzuki M, Inoki K, Sano Y, Sotozono C, Kinoshita S (2003) Successful regrafting of cultivated corneal epithelium using amniotic membrane as a carrier in severe ocular surface disease. Cornea 22:70–71
Nakamura T, Inatomi T et al (2006) Transplantation of autologous serum-derived cultivated corneal epithelial equivalents for the treatment of severe ocular surface disease. Ophthalmology 113:1765–1772
Nelson DJ (1988) Impression cytology. Cornea 7:71–81
Nishida K, Yamato M et al (2004) Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N Engl J Med 351:1187–1196
Nishiwaki-Dantas CM, Dantas PEC et al (2001) Ipsilateral limbal translocation for treatment of partial limbal deficiency secondary to ocular alkali burn. Br J Ophthalmol 85:1031–1033
Park KS, Lim CH et al (2006) The side population cells in the rabbit limbus sensitively increased in response to the central cornea wounding. Invest Ophthalmol Vis Sci 47:892–900
Pauklin M, Steuhl KP et al (2009) Characterization of the corneal surface in limbal stem cell deficiency and after transplantation of cultivated limbal epithelium. Ophthalmology 116:1048–1056
Pellegrini G, Traverso CE et al (1997) Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet 349:990–993
Puangsricharern V, Tseng SC (1995) Cytologic evidence of corneal disease with limbal stem cell deficiency. Ophthalmology 102:1476–1485
Rama P, Bonini S et al (2001) Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. Transplantation 72:1478–1485
Rao SK, Rajagopal R et al (1999) Limbal autografting: comparison of results in acute and chronic phases of ocular surface burns. Cornea 18:164–171
Sangwan VS, Tseng SC (2001) New perspectives in ocular surface disorders. An integrated approach for diagnosis and management. Indian J Ophthalmol 49:153–168
Sangwan VS, Vemuganti GK et al (2003) Successful reconstruction of damaged ocular outer surface in humans using limbal and conjuctival stem cell culture methods. Biosci Rep 23:169–174

Sangwan VS, Matalia HP et al (2005) Early results of penetrating keratoplasty after cultivated limbal epithelium transplantation. Arch Ophthalmol 123:334–340
Sangwan VS, Matalia HP et al (2006) Clinical outcome of autologous cultivated limbal epithelium transplantation. Indian J Ophthalmol 54:29–34
Schermer A, Galvin S et al (1986) Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol 103:49–62
Schwab IR, Reyes M et al (2000) Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. Cornea 19:421–426
Shimazaki J, Shimmura S et al (2002) Randomized clinical trial of deep lamellar keratoplasty vs penetrating keratoplasty. Am J Ophthalmol 134:159–165
Shimazaki J, Konomi K et al (2006) Ocular surface reconstruction for thermal burns caused by fireworks. Cornea 25:139–145
Shortt AJ, Secker GA et al (2008) Ex vivo expansion and transplantation of limbal epithelial stem cells. Ophthalmology 115:1989–1997
Solomon A, Ellies P et al (2002) Long-term outcome of keratolimbal allograft with or without penetrating keratoplasty for total limbal stem cell deficiency. Ophthalmology 109:1159–1166
Srinivasan BD, Eakins KE (1979) The reepithelialization of rabbit cornea following single and multiple denudation. Exp Eye Res 29:595–600
Theng JT, Tan DT (1997) Combined penetrating keratoplasty and limbal allograft transplantation for severe corneal burns. Ophthalmic Surg Lasers 28:765–768
Thoft RA, Friend J (1983) The X, Y, Z hypothesis of corneal epithelial maintenance. Invest Ophthalmol Vis Sci 24:1442–1443
Thompson HW, Malter JS et al (1991) Flow cytometry measurements of the DNA content of corneal epithelial cells during wound healing. Invest Ophthalmol Vis Sci 32:433–436
Tsai RJ, Li L et al (2000a) Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells(1). Am J Ophthalmol 130:543
Tsai RJ, Li LM et al (2000b) Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. N Engl J Med 343:86–93
Tseng SC (1985) Staging of conjunctival squamous metaplasia by impression cytology. Ophthalmology 92:728–733
Tseng SC, Prabhasawat P et al (1998) Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. Arch Ophthalmol 116:431–441
Tsubota K, Satake Y et al (1999) Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation. N Engl J Med 340:1697–1703
Van Buskirk EM (1989) The anatomy of the limbus. Eye 3:101–108
Vemuganti GK, Kashyap S et al (2004) Ex-vivo potential of cadaveric and fresh limbal tissues to regenerate cultured epithelium. Indian J Ophthalmol 52:113–120
Vemuganti GK, Fatima A et al (2009) Limbal stem cells: application in ocular biomedicine. Int Rev Cell Mol Biol 275:133–181
Williams KA, Brereton HM et al (1995) Use of DNA polymorphisms and the polymerase chain reaction to examine the survival of a human limbal stem cell allograft. Am J Ophthalmol 120:342–350
Zhou M, Li XM, Lavker RM (2006) Transcriptional profiling of enriched populations of stem cells versus transient amplifying cells. A comparison of limbal and corneal epithelial basal cells. J Biol Chem 281:19600–19609
Zieske JD, Bukusoglu G et al (1992) Characterization of a potential marker of corneal epithelial stem cells. Invest Ophthalmol Vis Sci 33:143–152

Chapter 30
Lacrimal Gland Regeneration: Progress and Promise

Shubha Tiwari and Geeta K. Vemuganti

Abstract The lacrimal gland is a tubuloacinar gland located in the groove of the frontal bone, which is involved in synthesis and secretion of major tear proteins and other aqueous components of the trilayered tear film. Any injury to the lacrimal gland, which may be age related, drug or radiation therapy induced leads to dysfunction of the gland with a resultant hyperosmolarity of the tear film and its subsequent instability. This tear film instability leads to destabilization of the ocular surface homeostasis and to a number of morbid complications like the dry eye syndrome.

The current treatment modality for chronic dry eye remains palliative, which provides only temporary symptomatic relief. One of the modalities of providing long-term benefit to these patients would be cell therapy to restore or replenish the damaged gland. This review describes the progress and promise of cell therapy for lacrimal gland regeneration for potential clinical application.

30.1 Introduction

The human lacrimal gland is a tubuloacinar, almond shaped gland located superior and lateral to the eye in the shallow depression of the frontal bone. The lacrimal gland can be divided into the main lacrimal gland (orbital and palpebral portions) and the accessory lacrimal glands (Gland of Wolfring and Krauss). The lacrimal

S. Tiwari, M.Pharm.
Sudhakar and Sreekant Ravi Stem Cell Biology Laboratory,
Prof Brien Holden Research Centre, L.V. Prasad Eye Institute,
Kallam Anji Reddy Campus, L.V. Prasad Marg, Hyderabad 500 034, Andhra Pradesh, India

G.K. Vemuganti, M.D., DNB, FAMS, FICP (✉)
School of Medical Sciences, University of Hyderabad,
Prof C R Rao Road, Gachibowli, Hyderabad 500 046, Andhra Pradesh, India
e-mail: deanmd@uohyd.ernet.in; gvmd@uohyd.ernet.in

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_30,

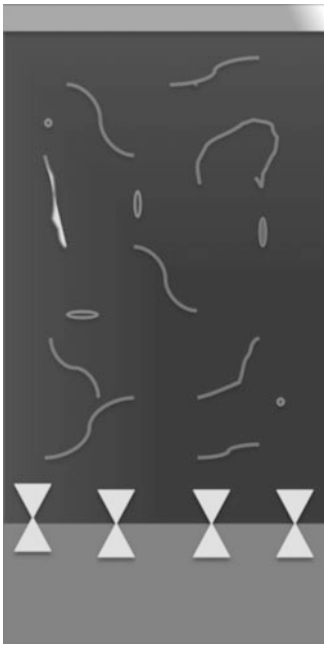


Fig. 30.1 Layers of tear film

gland forms an important entity of the lacrimal functional unit (LFU), which comprises of the lacrimal gland, the ocular surface (cornea, conjunctiva and the meibomian gland) and the sensory and motor nerves that connect them. The LFU controls the secretion of the major components of the tear film and is overall responsible for maintaining the stability of the tear film, transparency of the cornea and the quality of the image projected onto the retina (The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop 2007).

Tear film is constituted of the secretions of the lacrimal gland, meibomian gland and the conjunctival goblet cells. It has three basic layers: aqueous, which is 3–8 μm thick and is composed of the secretions of the lacrimal gland; lipid, which is 0.2 μm thick and is secreted by the meibomian gland; and the mucin layer, 1 μm thick, secreted mainly by the conjunctival goblet cells (Fig. 30.1). The important constituents of human tear are electrolytes like sodium, potassium, calcium, magnesium, bicarbonate and chloride; and major proteins like lysozyme, lipocalin, lactoferrin, scIgA, albumin and IgG (Table 30.1). Other components of tear film include lipids like phosphatidylcholine and phosphatidylethanolamine; mucins like MUC4, MUC5AC, MUC1. Minor components like defensins, catalase cytokines also form a part of the tear film composition (Tiffany 2008).

The tear film also contributes to the transparency of the cornea and determines the quality of image projected on the retina for cortical sensing. The periocular tear film is also responsible for providing nutrition to the cornea by acting as a coupling medium for the environmental oxygen; protecting the ocular surface due to the

Table 30.1 Composition of normal human tears

Composition	Concentration
Electrolytes	mmol/l
Sodium	128.7
Potassium	17
Calcium	0.32
Magnesium	0.35
Bicarbonate	12.4
Chloride	141.3
Major proteins	mg/l
Lysozyme	2.07
Lactoferrin	1.65
scIgA	1.93
Lipocalin	1.55
Albumin	0.04
IgG	0.004

antimicrobial properties of lysozyme, lactoferrin and lipocalin present; and also providing physical protection to the ocular surface against the shearing force of blinking due to the mucins present in it (Tiffany 2008).

30.2 Embryology and Development

The development of the human lacrimal gland has been the subject of numerous studies since the early 1900s. Most of these studies, other than the one published by Tripathi and Tripathi (1990), report that the gland develops from the ectoderm of the superior conjunctival fornix in human embryos with a crown to rump length of 22–24 mm (de la Cuadra-Blanco et al. 2003).

The two main lacrimal gland lobes – the orbital and the palpebral lobes – originate not simultaneously but one after the other. The orbital lobe originates from the proliferation of conjunctival fornix epithelial cells in the form of five or six epithelial buds and its formation concludes by the end of the second month. This is followed by initiation of the palpebral lobe formation. The orbital and the palpebral lobes are separated by the levator muscle tendon, which forms during the third week of development.

Epithelial-mesenchymal interaction, with its associated cell signaling, has been considered by a number of authors to be responsible for morphogenesis, organogenesis, cell differentiation and growth (Sanders 1988; Martin 1998; Makarenkova et al. 2000). Lacrimal gland development is an example of such an interaction in which the bud-like invagination of conjunctival epithelium at the fornix is the process, which initiates lacrimal gland development (Kammandel et al. 1999). The mesenchymal cells surrounding the point of epithelial budding are the periocular cells of

neural crest origin (Johnston et al. 1979). The tubular invaginations of the lacrimal gland extends and branches multiple times to form the lobular structure of the mature lacrimal gland.

30.3 Histology, Anatomy and Physiology

The lacrimal gland is a tuboacinar gland that consists of secretory epithelium arranged in a lobular pattern. These secretory acinar cells empty their secretions into ducts that anastomose into a larger nasolacrimal (NLD) duct which drains onto the ocular surface. Enveloping the secretory acinar cells are myoepithelial cells that contract and squeeze them facilitating the draining of the secretory components into the ducts. Between the lacrimal lobes are fibroblasts, which produce the collagen and matrix of interstitial regions, and mast cells, which secrete histamine and heparin (Fig. 30.2). In addition to this basic tissue architecture, the lacrimal gland is highly inundated with trafficking B and T lymphocytes as well as plasma cells (Walcott 1998).

The secretory acinar cells of the gland are columnar epithelium with basally located nucleus and a large perinuclear Golgi body. The ductal cells are more cuboidal in shape. The apical portion of the acinar and ductal cells has a number of vesicles and the cell base has an associated basement membrane that imparts the cells their polarity. Large junctional complex is found near the luminal pole that couples these cells electrically and chemically as well as mechanically attaches them with each other. Gap junctions like connexin 26 and 32 are also found here. The presence of a large number of junctional complexes between the epithelial cells indicate that these cells are very closely associated with each other (Walcott 1998).

The lacrimal gland is innervated by the sympathetic as well as the parasympathetic arms of the autonomic nervous system (Matsumoto et al. 1992). These nerves have a large number of cholinergic fibers and fewer adrenergic fibers. The parasympathetic

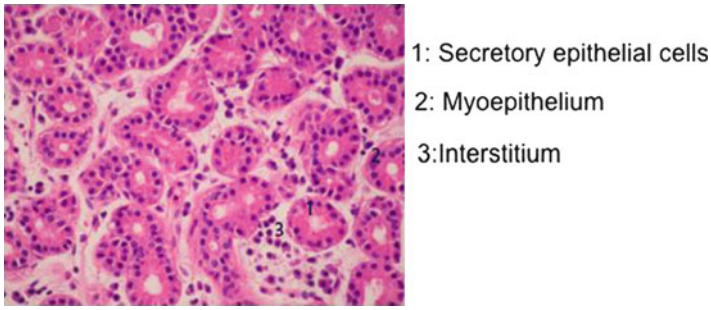


Fig. 30.2 Histology of normal human lacrimal gland *1* Secretory epithelial/acinar cells that synthesize and secrete major tear proteins. *2* Myoepithelial cells that envelope the acinar cells. *3* Interstitium that has fibroblasts that secretes collagen and other extracellular matrix

postganglionic neural cell bodies are found in the pterygopalatine (sphenopalatine) ganglion as well as the ciliary ganglion. Sympathetic fibers arise in the superior cervical ganglion. There is also some amount of sensory innervation of the gland from the trigeminal ganglia (van der Werf et al. 1996). Even though the innervation is similar across different species yet the nature and pattern of innervations as well as the pathway from these ganglia to the gland vary significantly from species to species.

The lacrimal gland secretes a number of proteins like lysozyme, lactoferrin, lipocalin, scIgA (Tiffany 2008). The secretion of these proteins is regulated by the nerves that innervate the gland and their associated nurotransmitters/neuropeptides (Walcott 1998). The important receptors present on the lacrimal gland are acetylcholine receptors like muscarinc M3 (Mauduit et al. 1993), vasoactive intestinal peptide type I and II, norepinephrine like alpha 1 and beta. Other receptors present are for neuropeptide Y, adenocorticotrophic hormone (ACTH) and alpha-melanocyte stimulating hormone. Since the epithelial cells of the gland are extensively coupled by junctional complexes, secondary messengers like inositol triphophate can easily diffuse between cells and activate the unstimulated cells too (Walcott 1998).

The muscarinic receptors in the gland are linked to G proteins, which are in turn linked to phospholipase C. This, on activation, releases inositol phosphate 3 (IP3) and diacyl glycerol (DAG) (Dartt 1989). IP3 induces the release of intracellular stores of calcium and opens calcium channels. DAG, on the other hand, activates protein kinase C isoenzymes, which further stimulates secretion. VIP receptors activate protein kinase A, which in turn causes cAMP release stimulating protein secretion (Hodges et al. 1997). Alpha adrenergic compounds cause protein secretion by activating protein kinase C (Walcott 1998).

Protein secretion in the acinar cells involves the fusion of vesicles with the apical membrane. There is also a basolateral membrane trafficking that is seen in these cells. This is responsible for the entry of molecules like prolactin into the cells. In addition, this basolateral membrane trafficking has also been implicated in antigen presentation and secretion of autoantigens (Mircheff et al. 1994) which leads to immune mediated apoptosis of acinar cells and loss of physiological function as seen in conditions like Sjogren's syndrome.

30.4 The Dry Eye Syndrome

The lacrimal gland forms an integral part of the lacrimal functional unit (LFU) due to its physiological function of secreting the aqueous component of the tear film. Any perturbation in the functioning of this gland, which may be age related, drug induced, autoimmune or due to orbital radiotherapy leads to destabilization of the tear film which in turn leads to a chronic debilating condition called the dry eye syndrome or keratoconjunctivitis sicca (KCS).

The International Dry Eye Workshop, 2007 (DEWS 2007) defined dry eye as:

> Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbances and tears film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of tear film and inflammation of the ocular surface.

The most important causative/contributing factors for dry eye are (DEWS 2007):

- Primary lacrimal gland dysfunction due to reduction in circulating androgens
- Secondary lacrimal gland dysfunction due to sarcoidosis, lymphoma etc.
- Autoimmune diseases like Sjogren's syndrome
- Reflex hyposecretion as in contact lens wear, diabetes, exposure to systemic drugs like antihistamines, beta blockers etc.
- Orbital radiotherapy for ocular malignancies
- Meibomian gland dysfunction

30.5 Etiology of Dry Eye Syndrome

Dry eye may be classified as aqueous deficient dry eye, caused due to lacrimal gland dysfunction; or evaporative dry eye, caused due to meibomian gland dysfunction. In the former, there is a deficiency in tear production itself due to perturbations in lacrimal gland function; and in evaporative dry eye, the rate of evaporation of tear from the ocular surface increases due to an unstable lipid film secreted by the damaged meibomian gland (DEWS 2007).

There has been a considerable increase in knowledge about the etiopathogenesis of dry eye syndrome in the past few years. The pathologic features of this condition include increased epithelial proliferation, stratification and abnormal differentiation with maintenance of a basal phenotype (Jones et al. 1998). This is accompanied by reduced expression of secretory and membrane-bound mucins by the ocular surface conjunctival epithelial cells (Danjo et al. 1998) compounding the effects of existing lacrimal dysfunction.

The two most important factors that contribute to the initiation and progression of dry eye are *tear hyperosmolarity* and *tear film instability* that adversely affects the ocular surface epithelial function and differentiation (Gilbard et al. 1989a, b). Trauma to a poorly lubricated and unprotected ocular surface due to blinking or environmental factors becomes a confounding factor, which worsens the condition (Lemp 1995; Danjo et al. 1998). Tear film stability, which is important in maintaining clear and sharp vision, is threatened when the interactions between stabilizing tear film constituents are compromised either by decreased tear secretion, delayed clearance, or altered tear composition as is seen in xerophthalmia and allergic eye diseases. Ocular surface inflammation is secondary consequence. Reflex tear secretion in response to ocular irritation is seen as the initial compensatory mechanism, but, with time, due to severe inflammation and chronic secretory dysfunction

a decrease in corneal sensation occurs which compromises the reflex response and results in even greater tear film instability. Dysfunction of the LFU is considered to play an important role in the evolution of different forms of dry eye (DEWS 2007).

Even though tear fluid is secreted as a hypotonic fluid, yet due to excessive evaporation from the exposed ocular surface or low rate of aqueous tear flow (or a combination of the two) tear hyperosmolarity may arise. This hyperosmolarity stimulates various inflammatory pathways involving the MAP kinases, inflammatory cytokines (interleukins, tumor necrosis factor alpha) and matrix metalloproteinases (MMP9). This cascade of events attracts the circulating T cells within the lacrimal glands. Under physiological conditions, the trafficking lymphocytes, finding no inflammation, would undergo apoptosis. However, in the presence of inflammatory signals, these lymphocytes become activated and secrete pro-inflammatory cytokines, which cause homing of additional T cells to the tissue and an increase in the level of inflammation thereby aggravating the existing condition and forming a vicious loop of inflammation (Fig. 30.3) (Gao et al. 1998). Any condition that results in hyper activity of the functional unit can also initiate inflammatory response within the lacrimal glands resulting in antigen presentation and cytokine secretion by the epithelial cells of the gland (Meggs 1993; Mircheff et al. 1998). These pro-inflammatory mediators cause epithelial cell death by apoptosis and also loss of conjunctival goblet cells – a combinatorial assault that leads to worsening of the existing condition.

30.6 Aqueous Deficient Dry Eye

In addition to release of inflammatory mediators, the etiology of dry eye also involves the loss of anti-inflammatory environment within the lacrimal glands, which may occur due to a drop in the levels of circulating androgens (Sullivan et al. 1984; Azzarolo et al. 1997). At the time of menopause in women, or due to various pathologic conditions, the level of circulating androgens may drop below a threshold level, thus making the tissues vulnerable for the initiation and progression of immune-based inflammation. A number of studies have shown a significant correlation between the levels of these inflammatory cytokines and the severity of ocular surface irritation symptoms, corneal fluorescein staining and the severity of conjunctival squamous metaplasia in patients (Pflugfelder et al. 1999).

Severe dry eye is also seen in patients of Sjogren's syndrome, which is an autoimmune disorder. In Sjogren's, autoantigens are expressed at the surface of the epithelial cells which causes homing and retention of tissue specific CD4 and CD8 cells. These lymphocytes cause loss of lacrimal acinar and ductal cells due to an immune mediated attack leading to tear hyposecretion and destruction of the gland. The precise trigger factors for Sjogren's is not known but risk factors include genetic profile, low androgen pool and certain viruses.

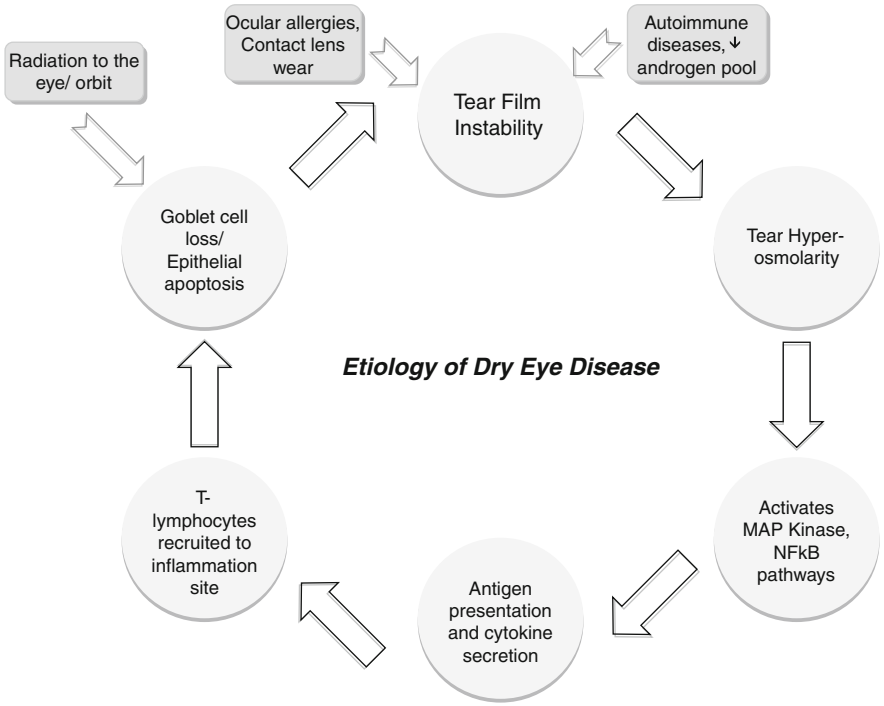


Fig. 30.3 Etiology of dry eye disease

Radiation therapy, which represents a commonly used modality in the treatment of ocular and oculoadenexal disorders including benign and malignant tumors, also contributes to the development of dry eye syndrome in patients. Despite a rapid evolution in the field of radiotherapy over the past years, a significant number of patients are still seen with acute and chronic ophthalmic complications including severe dry eye (Alberti 1997; Durkin et al. 2007). Preliminary data from our institute indicates that chronic dry eye develops in over 49 % of the patients who undergo external beam radiation therapy for ocular malignancies (unpublished data).

The causes of dry eye post-radiotherapy are multifactorial: (a) decrease in the lacrimal secretion leading to loss of aqueous layer; (b) ocular surface damage with goblet cell loss leading to loss of mucin secretion; (c) meibomian gland atrophy with loss of lipid layer secretion. In the early post-radiotherapy phase, lacrimal gland loss has been shown to be due to inflammation mediated apoptotic loss of acinar cells. In contrast, stem cells becoming sterile is proposed to be the main cause in late phase resulting in insufficient replacement of acinar cells with resultant decrease in tear secretion. This combined with the radiation induced fibrosis leads to loss of lacrimal function with progressive decrease in tear volume and finally dry eye (Stephens et al. 1991; Parsons et al. 1996; Barabino et al. 2005; Konings et al. 2005).

Contact lens wear is yet another condition that may lead to the development of severe dry eye in long-term users. The mechanism underlying the development of dry eye in these patients is probably the reduction in corneal sensitivity and an increase in tear film osmolarity due to chronic usage. Similar arguments have been proposed for the development of dry eye post-LASIK therapy.

30.7 Current Therapies for KCS

Even though there has been significant advancement in the knowledge about lacrimal gland dysfunction and development of dry eye syndrome, yet the same cannot be said for the management of the condition. Dry eye is still a chronic debilating condition, the treatment and management of which aims at palliation and improving the quality of life of the patient. The current treatment modalities available are lubricating agents like hydroxymethylcellulose, solutions containing bicarbonates and potassium, hyposmotic artificial tears (Hypotears, Novartis Ophthalmics) and artificial serum. In cases of severe dry eye, therapies such as anti-inflammatory medications (cyclosporins A, corticosteroids), pharmacological tear stimulants like diquafosol, rebamipide, ecabet sodium, pilocarpine etc. are employed. In certain instances, where the patient does not get any relief in symptoms by these, surgical interventions like punctal occlusion and salivary gland autotransplantation are done to slow down the progress of the condition (Management and therapy of dry eye disease: report of the Management and Therapy Subcommittee of the International Dry Eye WorkShop 2007).

On recommendation of the sub-committee on the therapy and management of dry eye, DEWS 2007 (Management and therapy of dry eye disease: report of the Management and Therapy Subcommittee of the International Dry Eye WorkShop 2007), the treatment/management protocol for this condition is now shifting towards employing strategies that would increase the natural production of tears, maintain ocular surface integrity and reduce/eliminate the levels of existing inflammation. With these objectives in mind various therapeutic avenues are being explored with the inclusion of cell therapy for restoring the damaged lacrimal gland.

30.8 Research on Lacrimal Gland

Given the sparsity of data on the etiopathogenesis and treatment of dry eye, it is still not clear how alteration in tear film composition can cause such a vicious cycle of tear film instability and chronic ocular inflammation. Even though a lot of research is being directed towards profiling the proteins, lipids and other constituents present in human tear yet there is a glaring lack of comparative data between normal individuals and dry eye patients.

30.8.1 Animal Studies

30.8.1.1 *In-Vitro* Cultures

An important area of investigation in this field is to find a common link between tear film osmolarity, tear film break up response and the resultant inflammatory stress. In order to facilitate these studies, not just *in-vivo* but also *in-vitro* models are being developed that would greatly assist the investigation into the secretory repertoire of lacrimal gland epithelia, regulation of secretion and etiopathogenesis of lacrimal gland conditions like Sjogren's syndrome.

Procedure for *in-vitro* culturing of lacrimal gland acinar cells has been evolving for nearly two decades now. Oliver et al. published one of the first reports on *in-vitro* culture of rat lacrimal gland acinar cells in 1987 wherein they described a method for culturing a dividing population of morphologically differentiated rat lacrimal acinar cells on a three-dimensional, reconstituted basement membrane gel. The cultured acinar cells proliferated on the basement matrix and showed the presence of cytoplasmic secretory granules (Oliver et al. 1987). However, their culture system could only maintain the epithelial cells for 6–7 days after which fibroblast overgrowth was observed. Successful *in-vitro* culture of lacrimal acinar cells was first achieved and published by Meneray and Rismondo in two separate reports in 1994 (Meneray et al. 1994; Rismondo et al. 1994). The importance of media formulation, supplement profile and extracellular matrix composition for optimal growth and functionality of these cells was first reported by Hann et al. (1991) and these findings were supported by a number of subsequent reports.

A major problem faced by all these investigators was that the lacrimal acinar cells could not be induced to proliferate significantly *in-vitro.* This issue was resolved by Schonthal et al., who reported in 2000 that the i*n-vitro* proliferation of lacrimal acinar cells could be improved significantly by the use of EGF, dihydrotestosterone (DHT), Matrigel and HepatoStim culture medium (Schonthal et al. 2000). Recent studies report the use of polyethersulfone dead-end tube (Long et al. 2006), denuded amniotic membrane (Schrader et al. 2007) as scaffolds and rotary cell culture system (Schrader et al. 2009) for successful *in-vitro* culture of rat or rabbit lacrimal glands.

The effect of androgen on the control of secretory component output by the lacrimal gland has been well established. The effect of androgens and androgen analogues on *in-vitro* culture of lacrimal acinar cells has helped elucidate the control that the androgens exert on the synthesis and secretion of secretory component (Sullivan et al. 1984, 1990; Hann et al. 1991; Kelleher et al. 1991) as well as other biochemical parameters related to the lacrimal secretion including the basal tear flow rate (Azzarolo et al. 1997).

The culture systems developed for the lacrimal acinar cells have also been optimized to assess the functionality of these cells. The currently employed conditions for the *in-vitro* culture of these secretory cells support the *in-viv*o mimicry of

their secretion pattern as elucidated by the detection of scIgA, lactoferrin, lysozyme, lacritin and a number of other tear proteins in the culture supernatant.

The last couple of years have seen an increase in the knowledge about the presence of stem-like cells in the lacrimal gland of mice (You et al. 2011), rat (Shatos et al. 2012) and humans (Tiwari et al. 2012). These studies indicate the inherent potential of the gland to heal itself following an insult. The study published by You et al. (2011) showed that post injection of interleukin into the mouse lacrimal gland which destroys areas in the gland, stem-like cells migrate towards the site of injury and heal the wound. These cells can be harvested and grown under *in-vitro* conditions too. However, the authors report minimum *in-vitro* growth from uninjured gland. In contrast, the recent study by Shatos et al.(2012) on rat lacrimal gland and our own experience with human lacrimal gland showed that stem-like cells are present in the native, uninjured gland too which can be maintained under appropriate *in-vitro* conditions.

The presence of stem cells in the lacrimal gland is an important finding that leads us to believe that these cells can be recruited to salvage the damaged gland. However, before we take a leap of faith the viability, homing and functionality of these cells need to be established by more extensive *in-vitro* studies and independent animal experimentation.

30.8.1.2 Animal Models

In order to better understand this condition, animal models have been developed which mimic the features of human dry eye syndrome. The important animal models that have increased our knowledge of this condition are:

Mouse models created using scopolamine and environmental dessicating stress show that osmolarity of tear as well as secretion of inflammatory cytokines is increased under such condition (Gilbard et al. 1989a, b; Stewart et al. 2005).

Mouse model of Sjogren syndrome dry eye have also been developed and it has been shown in these models that androgens have the potential to reduce the inflammatory response due to autoantigen presentation.

Neurturin deficient mice that develop dry eye and show elevated levels of inflammatory mediators in their tears (Song et al. 2003).

Rabbit model of KCS created using the technique of ablation, which shows that steroids like dexamethasone can be used to reverse the ocular surface damage and also to increase the low tear film break up time (Nagelhout et al. 2005).

Even though it is undeniable that these animal models have indeed increased our basic understanding behind the etiopathology of dry eye, yet the fact remains that the extent to which these animal models mimic the human condition is not clear. In order to bridge this gap in understanding the similarity/differences between the animal form and the human form of the dry eye condition, it is imperative that

Table 30.2 *In-vitro* lacrimal gland research: Information matrix

Year	Species	*In-vitro* research	References
1987	Rat	Established culture	Oliver et al. (1987)
1991	Rat	Importance of media & growth factors for in-vitro cultures	Hann et al. (1991)
1994	Rabbit	Physiologically responsive *in-vitro* cultures	Menerey et al. (1994) Rismondo et al. (1994)
2000	Rabbit	Purified acinar cell preparation	Guo et al. (2000)
2000	Rabbit	Use of EGF, DHT, Matrigel and HepatoSTIM for culture	Schonthal et al. (2000)
2000	Human	*In-vitro* culture from cadaveric tissue	Yoshino (2000)
1984	Rat	Effect of androgen on synthesis and secretion by lacrimal gland	Sullivan et al. (1984, 1990)
1990			Hann et al. (1991)
1991			Kelleher et al. (1991)
2006	Rat	*In-vitro* culture in polyethersulfone dead end tube	Long et al. (2006)
2007	Rabbit	*In-vitro* culture on amniotic membrane	Schrader et al. (2007)
2009	Rabbit	Rotary cell culture system	Schrader et al. (2009)
2011	Mouse	Report of mesenchymal stem cells in lacrimal gland post injury	You et al. (2011)
2012	Human	Established functionally viable cultures from fresh tissue, Preliminary report on presence of stem cells in native human lacrimal gland	Tiwari et al. (2012)
2012	Rat	Progenitor cells in uninjured rat lacrimal gland	Shatos et al. (2012)

studies be conducted on human tissue. Since it is unethical to undertake such studies without sufficient background information, *in-vitro* models of human lacrimal gland become very important tool for research.

30.8.2 Human Lacrimal Gland In-Vitro Cultures

In-vitro work on human lacrimal gland cultures is scarce, possibly due to the difficulty in obtaining human tissue for research. To the best of our knowledge and literature search, there is just one report published by Yoshino in 2000 (Yoshino 2000), which dealt with establishing human lacrimal cultures from cadaveric tissue. However, the study only reported the potential of these cells to secrete lactoferrin (Table 30.2).

Our group initiated work on human lacrimal gland cultures and reported the establishment of functionally viable human lacrimal gland *in-vitro* culture system

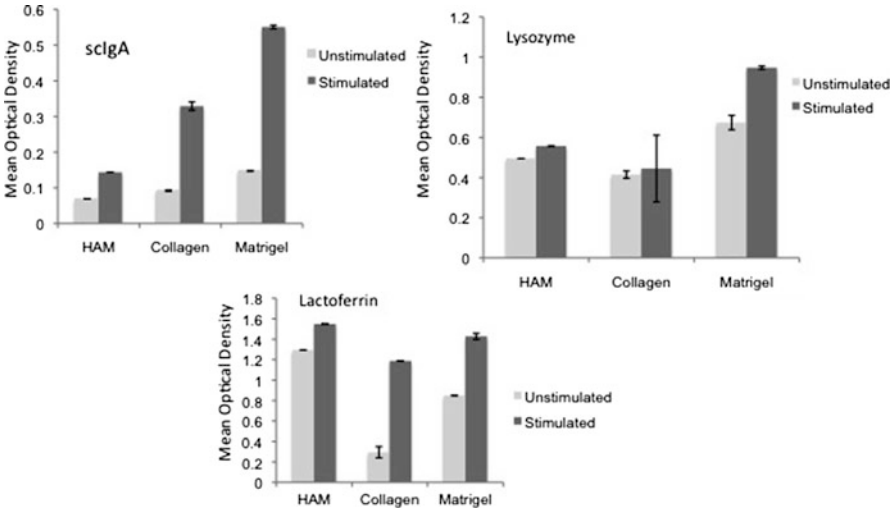


Fig. 30.4 Tear protein secretion by in-vitro human lacrimal gland cultures pre and post carbachol stimulation (Mean optical values can be correlated to protein levels)

from fresh exenteration specimens (Tiwari et al. 2012). The study also evaluated the growth of these cells on three matrices: Matrigel, collagen and denuded human amniotic membrane. The cultured cells showed three distinct morphologies: the cells with cobblestone epithelial morphology showed positivity for epithelial markers like CK3/12 and E-cadherin, the spindle shaped fibroblasts were positive for CD90 and vimentin; and cells with a 'whorled' epitheloid morphology were positive for myoepithelial markers GFAP and S-100. One of the important contributions of this paper is the evaluation of the conditioned media, which provides evidence that the cultured cells synthesize and secrete quantifiable levels of major tear proteins (statistically significantly more than negative controls) like scIgA, lactoferrin and lysozyme into the culture supernatant (Fig. 30.4). These cells could be maintained *in-vitro,* with intact secretory function, for a minimum period of 21 days. The cells show maximum levels of protein secretion by day 14 and then a decline over a period of time as expected. In addition, by day 16–18, these *in-vitro* cultures show the appearance of spherules and structures that look like ductal connections between them. Though further studies are warranted, the preliminary evidence points towards *in-vitro* gland formation.

Towards the long-term goal of cell therapy in chronic dry eye condition, it would be important to evaluate if these cells could be sustained. Using FACS and immunocytochemistry, we observed presence of stem-like cells (ABCG2 positive, ALDH high) in the native human lacrimal gland as well as in our *in-vitro* cultures. The cell suspension obtained from native gland (prior to culturing) show

3.1 ± 0.61% to be positive for the stem cell marker ABCG2. The number of cells that show ABCG2 positivity by day 14 is 0.3 ± 0.15%, which decreases slightly to 0.2 ± 0.13% by day 21.

30.9 Conclusion

Dry eye can be an extremely debilitating condition with high incidence of associated morbidity. The current prevalence of dry eye in the world is estimated at around 11–22% (Abelson et al. 2009). In the Indian context, these numbers are estimated to be around 18.4–20% (Sahai and Malik 2005; Gupta et al. 2008). These epidemiological numbers are a good indicator of the need for research on dry eye syndrome.

Even though we have come a long way in managing the chronic dry eye patients and improving their quality of life, yet there are many existing gaps in literature and a lot more needs to be done for these patients therapeutically. Large, well-defined, staged and age-matched studies that provide further insights into pathobiology of the disease progression and pinpoint to predictive biomarkers in tears would pave way for specific treatment.

A lot of what we know of KCS today has been by correlating animal data to human scenario. In order to lend credibility to the accrued knowledge, it is essential that a comparative study be made between human and mouse/rabbit tears and ocular surface protein- lipid profiles. This would enable us to identify the common components and pathways involved in various forms of this disorder and would also give important clues about the treatment of this condition.

Finally, restoration of physiological function can possibly be achieved by replenishing the stores of damaged tissue, hence cell therapy for chronic cases of dry eye appears a promising alternative.

Acknowledgements The authors wish to thank the patients who donated their exenterated tissue for research, the surgical oncology team of Dr Santosh G. Honavar, Dr Milind N.Naik, Dr Javed Ali and Dr Vijay Anand Reddy. We also thank the Director of Research, LVPEI, Dr D. Balasubramanian, for his constant support and encouragement.

Method of literature search

A search of Pubmed database (1979–till date) was conducted. Medline, Elsevier-EMBASE and Ophthalmic literature databases was also searched. The following key words were used: *Lacrimal gland/lacrimal in-vitro studies/dry eye syndrome.*

Additional sources include review of publications cited in other articles. Google search was also used to find publication that may be missed in the above databases.

Funding Agencies: International Atomic Energy Agency (IAEA), Department of Biotechnology (DBT), Hyderabad Eye Research Foundation (HERF), Champalimaud Translational Research Centre (C- Tracer), Sudhakar and Sreekanth Ravi Brothers

Financial Disclosure: None

References

Abelson MB, Ousler GW 3rd, Maffei C (2009) Dry eye in 2008. Curr Opin Ophthalmol 20(4):282–286

Alberti W (1997) Acute and late side effects of radiotherapy for ocular disease: an overview. Front Radiat Ther Oncol 30:281–286

Azzarolo AM, Mircheff AK, Kaswan RL et al (1997) Androgen support of lacrimal gland function. Endocrine 6(1):39–45

Barabino S, Raghavan A, Loeffler J et al (2005) Radiotherapy-induced ocular surface disease. Cornea 24(8):909–914

Danjo Y, Watanabe H, Tisdale AS et al (1998) Alteration of mucin in human conjunctival epithelia in dry eye. Invest Ophthalmol Vis Sci 39(13):2602–2609

Dartt DA (1989) Signal transduction and control of lacrimal gland protein secretion: a review. Curr Eye Res 8(6):619–636

de la Cuadra-Blanco C, Peces-Pena MD, Merida-Velasco JR (2003) Morphogenesis of the human lacrimal gland. J Anat 203(5):531–536

Durkin SR, Roos D, Higgs B et al (2007) Ophthalmic and adnexal complications of radiotherapy. Acta Ophthalmol Scand 85(3):240–250

Gao J, Schwalb TA, Addeo JV et al (1998) The role of apoptosis in the pathogenesis of canine keratoconjunctivitis sicca: the effect of topical Cyclosporin A therapy. Cornea 17(6): 654–663

Gilbard JP, Rossi SR, Heyda KG (1989a) Ophthalmic solutions, the ocular surface, and a unique therapeutic artificial tear formulation. Am J Ophthalmol 107(4):348–355

Gilbard JP, Rossi SR, Heyda KG (1989b) Tear film and ocular surface changes after closure of the meibomian gland orifices in the rabbit. Ophthalmology 96(8):1180–1186

Guo Z, Azzarolo AM, Schechter JE et al (2000) Lacrimal gland epithelial cells stimulate proliferation in autologous lymphocyte preparations. Exp Eye Res 71(1):11–22

Gupta N, Prasad I, Himashree G et al (2008) Prevalence of dry eye at high altitude: a case controlled comparative study. High Alt Med Biol 9(4):327–334

Hann LE, Kelleher RS, Sullivan DA (1991) Influence of culture conditions on the androgen control of secretory component production by acinar cells from the rat lacrimal gland. Invest Ophthalmol Vis Sci 32(9):2610–2621

Hodges RR, Zoukhri D, Sergheraert C et al (1997) Identification of vasoactive intestinal peptide receptor subtypes in the lacrimal gland and their signal-transducing components. Invest Ophthalmol Vis Sci 38(3):610–619

Johnston MC, Noden DM, Hazelton RD et al (1979) Origins of avian ocular and periocular tissues. Exp Eye Res 29(1):27–43

Jones DT, Monroy D, Ji Z et al (1998) Alterations of ocular surface gene expression in Sjogren's syndrome. Adv Exp Med Biol 438:533–536

Kammandel B, Chowdhury K, Stoykova A et al (1999) Distinct cis-essential modules direct the time-space pattern of the Pax6 gene activity. Dev Biol 205(1):79–97

Kelleher RS, Hann LE, Edwards JA et al (1991) Endocrine, neural, and immune control of secretory component output by lacrimal gland acinar cells. J Immunol 146(10):3405–3412

Konings AW, Coppes RP, Vissink A (2005) On the mechanism of salivary gland radiosensitivity. Int J Radiat Oncol Biol Phys 62(4):1187–1194

Lemp MA (1995) Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. CLAO J 21(4):221–232

Long L, Liu Z, Wang T et al (2006) Polyethersulfone dead-end tube as a scaffold for artificial lacrimal glands in vitro. J Biomed Mater Res B Appl Biomater 78(2):409–416

Makarenkova HP, Ito M, Govindarajan V et al (2000) FGF10 is an inducer and Pax6 a competence factor for lacrimal gland development. Development 127(12):2563–2572

Management and therapy of dry eye disease: report of the Management and Therapy Subcommittee of the International Dry Eye WorkShop (2007) Ocul Surf 5(2):163–178

Martin GR (1998) The roles of FGFs in the early development of vertebrate limbs. Genes Dev 12(11):1571–1586
Matsumoto Y, Tanabe T, Ueda S et al (1992) Immunohistochemical and enzymehistochemical studies of peptidergic, aminergic and cholinergic innervation of the lacrimal gland of the monkey (Macaca fuscata). J Auton Nerv Syst 37(3):207–214
Mauduit P, Jammes H, Rossignol B (1993) M3 muscarinic acetylcholine receptor coupling to PLC in rat exorbital lacrimal acinar cells. Am J Physiol 264(6 Pt 1):C1550–C1560
Meggs WJ (1993) Neurogenic inflammation and sensitivity to environmental chemicals. Environ Health Perspect 101(3):234–238
Meneray MA, Fields TY, Bromberg BB et al (1994) Morphology and physiologic responsiveness of cultured rabbit lacrimal acini. Invest Ophthalmol Vis Sci 35(12):4144–4158
Mircheff AK, Gierow JP, Wood RL (1994) Traffic of major histocompatibility complex class II molecules in rabbit lacrimal gland acinar cells. Invest Ophthalmol Vis Sci 35(11):3943–3951
Mircheff AK, Gierow JP, Yang T et al (1998) Sjogren's autoimmunity: how perturbation of recognition in endomembrane traffic may provoke pathological recognition at the cell surface. J Mol Recognit 11(1–6):40–48
Nagelhout TJ, Gamache DA, Roberts L et al (2005) Preservation of tear film integrity and inhibition of corneal injury by dexamethasone in a rabbit model of lacrimal gland inflammation-induced dry eye. J Ocul Pharmacol Ther 21(2):139–148
Oliver C, Waters JF, Tolbert CL et al (1987) Growth of exocrine acinar cells on a reconstituted basement membrane gel. In Vitro Cell Dev Biol 23(7):465–473
Parsons JT, Bova FJ, Mendenhall WM et al (1996) Response of the normal eye to high dose radiotherapy. Oncology (Williston Park) 10(6):837–847, discussion 847–838, 851–832
Pflugfelder SC, Jones D, Ji Z et al (1999) Altered cytokine balance in the tear fluid and conjunctiva of patients with Sjogren's syndrome keratoconjunctivitis sicca. Curr Eye Res 19(3):201–211
Rismondo V, Gierow JP, Lambert RW et al (1994) Rabbit lacrimal acinar cells in primary culture: morphology and acute responses to cholinergic stimulation. Invest Ophthalmol Vis Sci 35(3):1176–1183
Sahai A, Malik P (2005) Dry eye: prevalence and attributable risk factors in a hospital-based population. Indian J Ophthalmol 53(2):87–91
Sanders EJ (1988) The roles of epithelial-mesenchymal cell interactions in developmental processes. Biochem Cell Biol 66(6):530–540
Schonthal AH, Warren DW, Stevenson D et al (2000) Proliferation of lacrimal gland acinar cells in primary culture. Stimulation by extracellular matrix, EGF, and DHT. Exp Eye Res 70(5):639–649
Schrader S, Wedel T, Kremling C et al (2007) Amniotic membrane as a carrier for lacrimal gland acinar cells. Graefes Arch Clin Exp Ophthalmol 245(11):1699–1704
Schrader S, Kremling C, Klinger M et al (2009) Cultivation of lacrimal gland acinar cells in a microgravity environment. Br J Ophthalmol 93(8):1121–1125
Shatos MA, Haugaard-Kedstrom L, Hodges RR et al (2012) Isolation and characterization of progenitor cells in uninjured, adult rat lacrimal gland. Invest Ophthalmol Vis Sci 53:2749–2759
Song XJ, Li DQ, Farley W et al (2003) Neurturin-deficient mice develop dry eye and keratoconjunctivitis sicca. Invest Ophthalmol Vis Sci 44(10):4223–4229
Stephens LC, Schultheiss TE, Price RE et al (1991) Radiation apoptosis of serous acinar cells of salivary and lacrimal glands. Cancer 67(6):1539–1543
Stewart P, Chen Z, Farley W et al (2005) Effect of experimental dry eye on tear sodium concentration in the mouse. Eye Contact Lens 31(4):175–178
Sullivan DA, Bloch KJ, Allansmith MR (1984) Hormonal influence on the secretory immune system of the eye: androgen control of secretory component production by the rat exorbital gland. Immunology 52(2):239–246
Sullivan DA, Kelleher RS, Vaerman JP et al (1990) Androgen regulation of secretory component synthesis by lacrimal gland acinar cells in vitro. J Immunol 145(12):4238–4244
The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (DEWS) (2007) Ocul Surf 5(2):75–92

Tiffany JM (2008) The normal tear film. Dev Ophthalmol 41:1–20
Tiwari S, Ali MJ, Balla MM et al (2012) Establishing human lacrimal gland cultures with secretory function. PLoS One 7(1):e29458
Tripathi BJ, Tripathi RC (1990) Evidence for the neuroectodermal origin of the human lacrimal gland. Invest Ophthalmol Vis Sci 31(2):393–395
van der Werf F, Baljet B, Prins M et al (1996) Innervation of the lacrimal gland in the cynomolgous monkey: a retrograde tracing study. J Anat 188(Pt 3):591–601
Walcott B (1998) The lacrimal gland and its veil of tears. News Physiol Sci 13:97–103
Yoshino K (2000) Establishment of a human lacrimal gland epithelial culture system with in vivo mimicry and its substrate modulation. Cornea 19(3 Suppl):S26–S36
You S, Kublin CL, Avidan O et al (2011) Isolation and propagation of mesenchymal stem cells from the lacrimal gland. Invest Ophthalmol Vis Sci 52(5):2087–2094

Chapter 31
The Development of a Stem Cell Therapy for Deafness

Nopporn Jongkamonwiwat and Marcelo N. Rivolta

Abstract Medicine is at the doorsteps of a phenomenal revolution, brought by the advances in the stem cell field and the development of new technologies to engineer cells and tissues into more complex organs. The promise of a true regenerative approach to organ damage and loss of function is closer than ever of becoming a reality. The auditory field is participating of these developments with high expectations. Since the cochlea is an organ of difficult access and with very limited regenerative capacity, conventional therapeutic approaches have failed and, currently, the only treatments available are in the form of hearing aids and cochlear implants. The potential restoration of hearing by the use of exogenous stem cells will offer a solution to a condition that has very limited options. In this chapter, we are reviewing the increasing volume of research on this emerging field and discussing the key elements that need to be developed further, in order to translate the basic science into a clinical reality.

31.1 Introduction

Hearing, one of our primordial senses, is crucial for communication, pleasure and awareness. The impact of a hearing deficit, especially during childhood, is huge. It could lead to problems with the development of speech and language having implications for social integration and affecting quality of life as a whole. According to 2005 estimates by the World Health Organization (WHO), 278 million people worldwide have moderate to profound hearing loss in both ears (www.who.int/mediacentre/factsheets/fs300/en/index.html).

N. Jongkamonwiwat, Ph.D. • M.N. Rivolta, M.D., Ph.D. (✉)
Centre for Stem Cell Biology and Department of Biomedical Sciences, University of Sheffield, Addison Bldg, Western Bank, S10 2TN Sheffield, UK
e-mail: m.n.rivolta@sheffield.ac.uk

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_31,

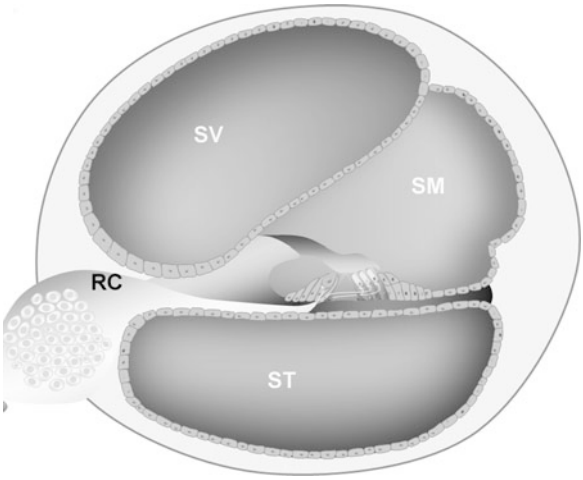


Fig. 31.1 Schematic illustration showing the anatomy of the cochlear duct. The cochlea comprises three fluid-filled chambers, separated in part by a bony structure, the osseous spiral lamina. The cochlear duct can be divided according to the type of fluid. The perilymph, contained in the Scala Vestibuli (*SV*) and Scala Tympani (*ST*) has an ionic composition similar to the extracellular medium, with high sodium concentration and low potassium. The Scala Media (*SM*) contains the endolymph, which in contrast to the perilymph, is high in potassium and low in sodium. The SM compartment harbours the organ of Corti, where the sensory hair cells play a crucial role in converting the mechanical sound wave into an electrical neural signal that is then sent to the auditory neurons. These auditory neurons have a bipolar morphology, with a dendrite that receives information from hair cells in the organ of Corti and the axon, which resides in the auditory nerve. The cell bodies of these neurons form the spiral ganglion, located in a discrete compartment, the Rosenthal's canal (*RC*)

Hearing impairment is most commonly caused by both inherited and acquired factors such as noise, prescription of ototoxic drugs or age (Holley 2005). The pathological changes underpinning hearing loss are concentrated mainly to the inner ear, which is the primary centre for hearing. The process of sound perception begins at the *cochlea*, a small organ situated within the temporal bone, which converts the mechanical sound wave into an electrical, nerve-transmitted signal. The cochlea contains two major types of sensory receptor cells, the inner hair cells (IHCs) and the outer hair cells (OHCs). The IHCs are the primary transducers, translating the displacement of their apical hair bundles, induced by the sound wave, into a depolarizing signal. The OHCs, on the other hand, receive efferent stimulus from higher control centres to modulate the auditory signal and contribute to magnify the sensitivity of the system (Webster 1992). The IHC signals are conveyed to higher auditory nuclei in the brainstem via the spiral ganglion neurons (SGNs), the primary order neuron of the auditory pathway (Fig. 31.1). There are several nuclei in the brainstem responsible for adjusting and tuning the signal from the cochlea before sending fibres for final interpretation of sound to the auditory cortex. The loss of sensory cells in the cochlea accounts for the majority of hearing

deficits (90%), and is classified as sensorineural hearing loss (SNHL). Such deafness can be caused by primary degeneration of the spiral ganglion neurons, in what is known as auditory neuropathy or by the primary loss of hair cells (Webster and Webster 1981; Hardie and Shepherd 1999). Secondary degeneration of neurons commonly follows HC loss and cell death occurs due to lack of trophic support (Fritzsch et al. 1997). However, in humans, this process is highly variable and depends on several factors (Nadol et al. 1989; Nadol 1997).

31.2 The Adult Mammalian Cochlea Does Not Regenerate Its Hair Cells or Neurons

While non-mammalian species can repair and heal their damaged sensory epithelia, the mammalian cochlea does not have the potential to regenerate neither the hair cells nor the sensory neurons (Raphael 2002). In avians and lower vertebrates, supporting cells (SCs) can be triggered by the signal of dying hair cells to replace them by either proliferating or transdifferentiating modes (Morest and Cotanche 2004). Just like in birds, the mammalian supporting cell shares a common progenitor with hair cells during development (Fekete et al. 1998). However SCs of the mammalian organ of Corti fail to show any regenerative response to HC loss, via either direct transdifferentiation or mitosis (Forge et al. 1998). The vestibular organ (including the utricle, the saccule and the cristae ampullaris) is also a mechanosensory structure located in the inner ear that conveys information on position and gravitational acceleration. A mild regeneration of hair cells has been observed in the vestibular sensory epithelia in guinea pigs following ototoxic drug treatment at different time-points (Forge et al. 1993). Recently, a study on the murine utricle has confirmed evidence that vestibular hair cells can spontaneously regenerate after exposure to the ototoxic drug gentamycin. Large number of immature hair cells could be seen as early as 2 weeks after the lesion. However, neither the regenerated cell numbers nor their appearance were normal (Kawamoto et al. 2009). Population of stem cells can be isolated from the adult mouse utricle (Li et al. 2003a), but the population virtually disappears from the mouse cochlea after the third week of age (Oshima et al. 2007). The proliferation and differentiation of mouse inner ear stem cells in vitro seems to be regulated by Notch signalling, just as the progenitors in vivo. Inhibition of Notch signalling by γ-secretase enhances hair differentiation by upregulating the expression of Atoh1. On the other hand, activation of Notch signalling by its intracellular domain (NICD) increases proliferation but also differentiation of neural phenotypes by upregulating Sox2 and the neuronal trancription factor Ngn1 (Jeon et al. 2011).

The mature vestibular and auditory organs differ greatly, not only in their ability to contain stem cells but also in their potential for regeneration. What is the underlying explanation for the differences of these two related, neighbouring sense organs? Part of the answer may lie in the fact that cochlear supporting cells become terminally postmitotic during embryogenesis whereas supporting

cell in the vestibular organ show a lingering proliferative capacity (Rubel et al. 1995). However, it is not clear whether the lack of mammalian hair cell regeneration could be due to an intrinsic loss of competence for SC to divide and differentiate, to the absence of appropriate mitogenic signals, or to the presence of instructive signals that actively block the regenerative capacity. The fact that proliferative stem cells cannot be isolated from the murine cochlea after the third week of age, despite of the presence of mitogens in the media and the removal from any potential inhibition present in the tissue, would suggest that changes are more likely intrinsic and fixed. Cell cycle regulators are believed to be key factors in maintaining the post-mitotic state of SCs. Upregulation of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor (CDKI) is found in the cochlear epithelium at the same time that terminal mitosis begins during embryogenesis, and it remains in the SC till the adulthood (Chen and Segil 1999). Evidence has been presented that a perinatal population of supporting cells can downregulate $p27^{Kip1}$ *in vitro* and re-enter the cell cycle. This ability is severely reduced by the time they reach to P14. However, even at this stage, a small proportion of cochlear supporting cells can transdifferentiate in vitro into hair cells-like cells (White et al. 2006). Supporting further the idea that the ability of these cells to proliferate is $p27^{Kip1}$-dependant, cells taken from null animals had an enhanced proliferative capacity in culture when compared with wild type-ones (White et al. 2006). The level of expression of $p27^{Kip1}$ protein remains robust in differentiated cochlear SCs *in vivo*, suggesting that $p27^{Kip1}$ imposes strong inhibition on cell mitosis in differentiated SCs and may prevent them from dividing after HC loss. The inhibition of SC proliferation seems to be a major factor that blocks the possibility of HC regeneration in the mammalian cochlea. Likewise, the SCs may loose their ability to convert directly into HCs over development. Experiments have shown that new HCs are generated when existing HCs are ablated in the mouse organ of Corti prior to E16, but this ability is lost after E16 (Kelley et al. 1995). Early postnatal rats treated with the ototoxic drug amikacin generated cells that had mixed features between hair and supporting cells. These cells have been interpreted as having attempted direct transdifferentiation but failing to achieve complete HC morphological characteristics (Daudet et al. 1998). Together, these findings suggest that the ability of auditory SCs to undergo direct transdifferentiation is limited over the developmental process. Mature SCs may have differentiated too far for functional, direct transdifferentiation to occur.

In contrast to cochlear HC, there is some evidence to suggest the presence of neural progenitor cells in the adult auditory nerve. Although Oshima et al. failed to isolate stem cells/progenitors from the spiral ganglion of adult mice (Oshima et al. 2007), Rask-Anderson et al. isolated nestin-positive neural progenitor that also expressed TrkB and TrkC from adult human and guinea pig spiral ganglion tissues (Rask-Andersen et al. 2005). Regeneration of SGN cannot be observed after degeneration; therefore damage to neurons can lead to permanent deafness (Sekiya et al. 2003). Even when the cell body and central axon survive, deafness can still occur due to degeneration of peripheral processes (Nadol 1997). SGN

degeneration has been described in a variety of pathologies. Exposure to sound pressure levels that do not harm HC and cause HC loss can still insult the SGN and trigger neuronal degeneration (Kujawa and Liberman 2006). In humans, the regrowth of SGNs does not appear to be clinically significant (Nadol 1997). In summary, although there are indications that some reparative potential is still present in the adult spiral ganglion, recovery does not seem to take place at any substantial rate after damage to HCs or neurons in the adult mammalian cochlea.

31.3 Prevention of Damage by Neurotrophic Factors

There have been numerous attempts to protect and prevent HCs and SGNs from degeneration triggered by drugs, noise or age by treatment with neurotrophic factors (NTFs) or exogenous reagents. The major exogenous compounds that have been applied to the auditory system are neurotrophin-3 (NT-3) and brain derived neurotrophic factor (BDNF). Neurotrophin secretion is reciprocal among HCs and SGNs (Duan et al. 2004). The production of NT-3 is crucial to the survival of type 1 SGNs during development of IHCs innervation whereas BDNF is required for SGN type 2 survival (Ernfors et al. 1995). Degeneration of adult SGNs can be prevented by infusion of NT-3 (Ernfors et al. 1996; Duan et al. 2000). These efforts have shown the benefit of NTFs supplementation in reducing the degeneration of SGNs secondary to HC loss, suggesting possible clinical improving for the cochlear implantation. It has been shown that infusion of a combination of BDNF and ciliary neurotrophic factor (CNTF) into the cochlea can enhance the survival of SGNs and also restore the evoked auditory brainstem response (eABRs) after chemically inducing deafness and mimicking the cochlear implantation by introducing a platinum-iridium electrode to deliver electrical stimulation (Shinohara et al. 2002). Recently, Agterberg et al. have shown a significantly improvement of eABR thresholds after BDNF treatment via osmotic pump 2 weeks after cessation of treatment in the HCs degeneration model induced by kanamycin (Agterberg et al. 2009). Other routes of administration (such as BDNF-embedded gelfoam applied to the round window) have been explored, with more modest sucess (Havenith et al. 2011). A potentially interesting development is the use of cells to deliver the necessary neurotrophins. This application should bypass the limited delivery time associated with the use of osmotic pumps. When BDNF-expressing Schwann cells, encapsulated in an alginate matrix, were inserted in the cochlea of deaf guinea pigs it showed enhanced survival of auditory neurons (Pettingill et al. 2011). Although NTFs increase the SGN survival after HC degeneration producing an enhancement of functional capability, they have not yet been tested in a real clinical condition and their application remains unproven. However, once their safety for human application is established, they would likely be of clinical utility as supportive treatment during cochlear implantation.

31.4 A Therapeutic Solution for Deafness: The Cochlear Implant

Giving the lack of a regenerative response in the mammalian ear, the development of a surgically implanted prosthesis was seen as the only medical effective therapy for hearing loss. The first attempts for cochlear implantation were performed in the 1950s by the collaboration between Djourno and Eryies, a French surgeon and an engineer, to place a coil of wire in the inner ear of two deaf patients. Although these trials failed after a short time, they kick-started an area of research that was going to deliver substantial advances (Eisen 2003). A modern cochlear implant is an electronic device that can be divided into two major parts: the external head piece that includes a microphone and speech processor and is placed on the skin close to the temporal bone area, and the internal cochlear electrode. The external component functions as a transmitter to process sound signals and is connected to the receiving coil which, secured on the temporal bone beneath the skin, is responsible to convert the signals into electric impulses and deliver them through an internal cable to the implanted electrode in the cochlea. An array of up to 22 electrodes wound up through the scala tympani of the cochlea stimulates the SGNs, which in turn sends the information to the brain via the auditory nuclei. The cochlear implant can give a quality of sound discrimination fine enough to understand speech but post-implantation rehabilitation is critical for ensuring the effectiveness of treatment. Modern cochlear implants allow the typical patient to understand more than 90% of words in unfamiliar sentences when presented in quiet listening conditions (Spahr and Dorman 2004). Normal criteria for implantation will require that the candidate be severe to profoundly deaf in both ears and SGNs will have to be preserved. In certain parts of the world, the high costs of the operation and prosthesis have a heavy influence in their accessibility. Because many patients were unable to benefit from cochlear implantation due to an auditory nerve dysfunction, this led to the development of the auditory brainstem implant (ABI). The principle of ABI is similar to cochlear implant but bypasses the function of SGNs by stimulating the cochlear nucleus (CN) directly via surface mounted 'button' electrodes (Moore and Shannon 2009). This requires an electrode implanted directly into the brainstem, making this device more risky and still not widely used. The best performance of ABI is still poor when compared with cochlear implantation (Moore and Shannon 2009).

31.5 Cellular Replacement: A Promising Therapeutic Strategy

Given the lack of endogenous regeneration and the limited therapeutic range available, the potential to develop a treatment based on the delivery of exogenous cells offers new hopes. Cell-based approaches have been proposed directed to the replacement or restoration of damaged HCs and/or SGNs. Stem cells are excellent candidates for biological implantation as they have the potential to proliferate

and differentiate, both required features for a regenerative strategy option. The optimisation of a cell transplantation strategy is a phenomenal task, since there are huge variables to consider in each experimental paradigm. The range of different stem cells and cell lines of potential use, the state of the host tissue and the routes for delivery are all issues that could affect the efficiency of transplantation. Stem cell transplantation experiments could also be excellent models in the context of drug discovery and development. They may be combined with supplemental treatments such as NTFs or exogenous compounds that could provide the niche for transplanted cell survival or, alternatively support differentiation into the target cell type. In the following sections, we will discuss the current evidence on the achievements and challenges lying ahead for stem cell-based therapeutic strategies for hair cell and spiral ganglion cell loss, emphasising on the different cell types and delivery routes used in each experimental condition.

31.6 Cell Candidates for Transplantation

Several stem cell types and lines have been used in transplantation experiments, ranging from directly transplanting undifferentiated embryonic (ESCs) and mesenchymal stem cells (MSCs) to various kinds of chemically differentiated lineages, including some genetically modified cells.

31.6.1 Embryonic Stem Cells

Most of the stem cells used so far in transplantation studies have been from murine origin. Many ESCs have also been genetically modified by tagging them with green fluorescent protein (eGFP), a reporter gene used for tracing after transplantation. In a study performed in deafened guinea pigs, undifferentiated and partially differentiated mouse embryonic stem (ES) cells were delivered into the cochleae (Hildebrand et al. 2005). Cells survived in the site of transplantation and were also observed in the cochlear chambers, stria vascularis, endolymph fluid of the scala media and spiral ligament for a postoperative period of at least 9 weeks. However, cells failed to differentiate. There was no evidence of significant immunological rejection of the transplanted cells, this being quite relevant since no apparent immunosuppressant treatment was provided. Sekiya et al. described extensive migration of mESCs in the cochlear modiolus after transplantation (Sekiya et al. 2006). Cells were observed not only in the cochlear nerve but were also present in the Rosenthal's canal and scala media. Although they appeared to migrate more extensively into the damaged auditory nerve than on undamaged ones, cells on intact nerves showed more signs of neuronal differentiation. In a different study, implanted ESCs done in parallel with embryonic dorsal root ganglion neurons have migrated to the area close to the ventral cochlear nucleus (Hu et al. 2004). Genetically modified mESCs

to conditionally express neurog1, have also been implanted into deafened animals. After a short induction in vivo with doxycycline, to induced transient transgene expression of neurog1, a continuous supplement of glial derived neurotrophic factor (GDNF) was provided for over 3 weeks (Reyes et al. 2008). The results showed the transplanted cells in several areas of the cochlea, cochlear modiolus, Rosenthal's canal and the site of transplantation, scala tympani. Surviving cells were found mainly in the scala tympani, they had more of a neuronal -like appearance and expressed the neuronal maker TUJ1 than cells in cochlear modiolus. Moreover, the majority of TUJ1 positive cells were co-labelled with the vesicular glutamatergic neuron marker VGLUT (Altschuler et al. 2008).

31.6.2 Neuroprogenitors Induced from Embryonic Stem Cells

Mouse ESCs were differentiated, before transplantation, into neuroectoderm-containing embryoid bodies (EBs) by a combination of basic fibroblast growth factor (bFGF) and insulin-transferrin-sodium selenite (ITSS). Unfortunately, low number of cells survived in both, the site of transplantation and the target area. Remaining cells showed the potential to undergo differentiation in the xenografted host by expressing both neuron and glia phenotypes (Coleman et al. 2006). In an independent study, EYFP ESC-derived neuroprogenitor cells were used in the ouabain-induced model of SGN damage. ESCs were exposed to bFGF and enriched for neuroprogenitor cells. Transplanted cells at the cochlear nerve trunk showed strong evidence of neuronal differentiation by extending their perpheral processes toward the organ of Corti. Cells survived for more than 3 months and formed abundant processes projecting through the Rosenthal's canal (Corrales et al. 2006). In a different approach, mESC-derived embryoid bodies were co-cultured with SGN or HC explants, isolated from P5 (postnatal day 5) rats. Hair cell explants co-cultured with embryoid bodies showed a significantly greater number of neurofilament-positive and neural-like cells (Coleman et al. 2007). More recently, mESCs induced into neural differentiation by retinoic acid (RA) were used to compare the effect of different periods of time between the onset of injury induction and transplantation (Lang et al. 2008). This experiment showed a significant difference in the survival of transplanted cells, with greater survival rates obtained when transplantation occurred soon after injury (i.e., 1–3 days after induction of damage). The ability of RA to produce sensory neural differentiation from mESC can be enhaced when combined with BMP4. However, although these neurons are sensory in nature (as measured by the expression of peripherin and Brn3a) there is no evidence that they express otic–specifc markers (Nayagam and Minter 2011).Most examples using mESC-derived cells aim to replace neurons. The replacement of HCs is still very difficult due to the limitations in the surgical techniques for cell delivery. Nevertheless, mESC have been explored in vitro with the aim to produce hair cell-like cells. Sensory cells were derived from mESCs by culturing them ina chemical defined media with several growth factors. The mESC-derived phenotypes expressed

HC markers, Atoh1, Brn3c and Myosin VIIa and a small proportion of them also showed HC morphology like stereocilia, which co-labelled with espin and phalloidin (Li et al. 2003b). The same group has now published an improved protocol that produces otic progenitors and then hair cell-like cells from mESCs. By culturing the progenitors with chicken-derived stroma cells, a robust differentation of the apical bundle was obtained, and more importantly, the cells expressed mechanotransduction currents (Oshima et al. 2010).

Early attempts to produce sensory neurons from human embryonic stem cells are also producing encouraging results. Human embryonic stem cells (hESCs) were induced to form embryoid bodies and latter transferred to differentiation media in the presence of NT-3, BDNF, FGF or bone morphogenetic protein 4 (BMP4) The hES derived neuroprogenitor cells showed projecting fibres to denervated *ex vivo* sensory epithelia and expressed synaptic markers. Moreover, the neuroprogenitor cells were transplanted in the cochlear nerve trunk of deafened animal. The transplanted cells engrafted in the auditory nerve trunk and sent out processes which grew toward the auditory sensory epithelium (Shi et al. 2007). Furthermore, functional and specific auditory sensory neurons have been produced from hESCs using a step-wise protocol that generates otic progenitors. Restoration of auditory evoked responses by human ES-cell-derived otic progenitors (Chen et al. 2012). These cells survive, differentiated and grow neurite projections when transplanted into deafened cochleae (Chen et al. 2012).

31.6.3 Neural Stem Cells (NSCs)

Neural stem/progenitor cells have also been used for otic transplantation. Neurospheres, obtained from adult mouse lateral ventricle, were introduced into either normal or deafened inner ears. These neural stem cells (NSCs) were transduced with Neurogenin 2 by retroviral transfection. Survival rate of transplanted cells was relatively poor, even in the neurogenin-transduced group. There was no significant difference in survival rate between deafened and normal animals. The transplanted cells distributed in the perilymphatic chamber and in the Rosenthal's canal (Hu et al. 2005b). Interesting results were obtained from Parker et al. using the c17.2 cell line, a NSC line derived from immortalized male murine foetal cerebellar cells and implanted into sound-damage model (Parker et al. 2007). The transplanted cells were traced by Y-chromosome fluorescence in situ hybridization (Y-FISH). NSCs were found with characteristic of both neural tissues (satellite, spiral ganglion and Schwann cells) and cells of the organ of Corti (hair cells and supporting cells). Although still a single report that may need further confirmation this study shows that, potentially, several lineages could be obtained from neural stem cells, besides the expected neurons and glia.

Recent studies on transplantation using NSCs were done in undamaged animal model to observe their distribution and potential effect on auditory function (Fu et al. 2009). NSCs were dissociated from hippocampal tissue of rat embryos and transferred

to neurophere culture media with the combination of bFGF and epidermal growth factor (EGF). Transplanted NSCs were able to survive in the perilymphatic space 2 weeks after transplantation, some of them were also observed in the endolymphatic space and Rosenthal canal. Unfortunately, there was no report of differentiation from this experiment. There was an experiment combining cell therapy with chronic electrical stimulation (CES) and exogenous neurotrophic growth factor (NGF) recently published by Hu et al. (2009). Embryonic dorsal root ganglion neurons (DRG) were implanted into deafened animal. Implanted DRG cells were found close to Rosenthal canal in the adult cochlea for up to 4 weeks after transplantation. They also shown an extensive neurite projections penetrating into the bony modiolus and reach the spiral ganglion region in animals supplied with CES and/or NGF.

31.6.4 Auditory Progenitor/Stem Cells

Tissue specific stem/progenitor cells are probably the cell type that more accurately resembles the process of normal differentiation that takes place in vivo. Although they may lack an extensive proliferative capacity, which could make difficult the scaling up of their production for therapeutic applications, their cochlear origin validates them as important systems for development and analysis. Several papers have reported the isolation of progenitor cells from either developing or early postnatal mouse cochleae. However, the lineage potential for the majority of these systems has only been established for one given phenotype. Many of these cells have been shown to produce *in vitro*, either HCs (Malgrange et al. 2002; Zhai et al. 2005; Oshima et al. 2007; Savary et al. 2007) or SGNs (Rask-Andersen et al. 2005), but rarely both. Interesting exceptions are the human foetal auditory stem cells (hFASCs), a population recently isolated from 9 to 11 week old human foetal cochleae and described below in detail (Chen et al. 2007, 2009a, b).

31.6.5 Mesenchymal Stem Cells

Only few studies have looked at mesenchymal stem cells (MSCs) transplanted into the cochlea. Mouse MSCs were delivered into the perilymphatic space in a xenograph host. Seven days post-operation, most transplanted MSCs were found in the scala tympani and scala vestibule, and only a small number located in the scala media. No GFP-positive MSCs were found in the cochlear modiolus (Matsuoka et al. 2006). In a later experiment, the same group compared a deafened model with a normal host. This experiment also studied the effect of different routes of transplantation, comparing between intraperilymphatic and cochlear modiolar injection. Transplantation into the perilymphatic duct was unable to deliver cells into the Rosenthal's canal. With the modiolar injection, undifferentiated MSCs were able to

survive both in the control and deafened cochlea. However, the average number of transplanted cells found in the modiolus was greater in the deafened ear than in control (Matsuoka et al. 2007). Unfortunately, there was no indication of differentiation of transplanted cells from either experiment. Although the replacement of sensory cells by MSCs remains to be demonstrated, they could be used to target different types of SNHL. In some cases, the original problem lies not with the sensory cells but in the stria vascularis, a tissue in the lateral part of the cochlear duct responsible for the control of potassium homeostasis and for the generation of the endocochlear potential. A model for SNHL has been developed that generates dysfunction of the fibrocytes critical for the normal flow of potassium ions in the endolymphatic compartment. The application of the mitochondrial toxin 3-nitropropionic acid (3NP) induces acute SNHL by selective degeneration of the fibrocytes of the cochlear lateral wall, without inducing any significant damage to HCs or SGNs (Kamiya et al. 2007; Kada et al. 2009). Using this model, bone marrow MSCs were allogeneic transplanted via the lateral semicircular canal. MSCs were observed to localize at the ampullary area close to the transplanted site and in the perilymphatic ducts. Transplanted MSCs cells were found to replace the fibrocyte at the cochlear lateral wall confirmed by co-localization of BrdU and connexin 26 markers. Moreover, a recovery of hearing was detected by using ABR measurements. There was no report for the distribution in any others area of the cochlea and also no evidence to show differentiation of MSCs into another cell lineage (Kamiya et al. 2007).

31.6.6 Induced Pluripotent Stem Cells

The generation of induced pluripotent stem cells (iPSCs) appears to hail a new era in stem cell research. The potential to produce patient-specific stem cells with properties resembling those of hESCs should have huge impact in the development of cell-based therapies. Cells could be applied as autologous transplants, bypassing the need for immunosupression. Moreover, iPSCs are not surrounded by the ethical concerns associated with the use of hESCs. By using a relatively 'simple' protocol, that is the forced expression of four critical pluripotency factors (*Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*) under ESC culture conditions, differentiated cells such as fibroblasts can be reprogrammed and turned into undifferentiated, ESC-like cells (Takahashi and Yamanaka 2006). Moreover, a very recent report demonstrated that generation of human iPSCs requires only induction by the Oct4 gene (Kim et al. 2009).

Although still a novel technique, an early report has started to explore its potential for the auditory system. Mouse iPSCs were neuralized by exposing them to stromal cell-derived inducing activity (SDIA), showing evidence of neuronal differentiation *in vitro*. Neuralized iPSCs were then transplanted into the cochlea. Differentiation was observed 1 week after transplantation. The iPS-derived neural progenitor cells survived and were able to project their neurites toward cochlear hair

cells. The marker for glutamatergic neuron was expressed in some of transplanted cells indicating the potential of differentiation in the host tissue (Nishimura et al. 2009). But more interstingly, using a combination of growth factors, cell signalling inhibitors and growing them over chicken stromal fibroblasts, Heller's group showed that mouse iPSCs can generate hair cell-like cells with the ability to mechanotransduce (Oshima et al. 2010).

A key factor regarding the different types of cells for transplantation is their stage of differentiation. The more undifferentiated ESCs seem to have greater potential than progenitor cells in terms of migration and distribution in the cochlea. Neuroprogenitor cells are more committed to undergo differentiation and shown higher capacity to generate neuronal-like cells in the host tissues. The transplantation of neuroprogenitor cells into the cochlear nerve trunk has so far, provided the most convincing evidence that transplanted cells can integrate into the host tissue and project processes toward the organ of Corti. However, the transplanted cells are generally established as an ectopic ganglion, remaining at the site of transplantation rather than migrating into the Rosenthal's canal. MSCs, on the other hand, show very strong propensity to differentiate into mesodermal cell types suggesting to be a promising therapy for patients with SNHL attributed to degeneration of cochlear fibrocytes. Nevertheless, migration and differentiation are not just dependent on the characteristics of transplanted cell types. Of critical importance is the technique for delivering them to the right place within the cochlea.

31.6.7 Human Fetal Auditory Stem Cells: A Model for Cochlear Stem Cell Biology in Humans

Despite the advances obtained in rodents, until recently hearing research has suffered from the lack of a suitable model to study stem cell biology of the auditory organ in humans. This started to change a couple of years ago when a population of stem cells was identified in the human fetal cochlea (Chen et al. 2007) and later, a protocol was developed that allowed their isolation and expansion in vitro (Chen et al. 2009a, b). By culturing dissociated cells from sensory epithelia from 9 to 11 weeks-old foetuses in a serum-free media supplemented with EGF, IGF1 and bFGF (and referred as OSCFM, *Otic Stem Cell Full Media*), an homogenous population that expressed stem cell markers such as NESTIN, SOX2, OCT4 and REX1, among others, was selectively expanded.

Several stem cell lines were established that retained expression of these stem cell markers and remained proliferative for several months. When cells were grown under defined culture conditions and passaged using a non-enzymatic protocol, the cells remained undifferentiated, growing as an adherent monolayer and displaying an epithelial-like morphology. However, when they were passaged using trypsin, neuronal differentiation was readily induced. Cells grew processes and elongated. After 24–36 h they displayed the characteristic bipolar morphology of spiral ganglion neurons. The differentiation process was further supported by exogenous

factors such as Shh, NT3 and BDNF, as measured by the expression of the neuronal markers *NEUROGEN1*, *BRN3A*, b-TUBULIN III and NEUROFILAMENT 200. Moreover, 5–7 days after inducing differentiation bipolar cells displayed potassium delayed rectifiers and voltage-gated sodium currents. These findings are important as some neurons obtained from mouse embryonic stem cells have failed to express sodium channels (Balasubramaniyan et al. 2004). On the other hand, culture in the presence of RA and EGF favoured the differentiation into hair cell-like phenotypes by inducing the expression of *ATOH1* and *BRN3C* as well as MYOSIN VIIA and PARVALBUMIN. Furthermore, these cells showed a rearrangement of the actin cytoskeleton, resembling the cuticular plate, and expressed the inward rectifier K^+ current (I_{K1}), whose slow decay and voltage activation range closely resembled those recorded in pre-hearing mouse cochlear hair cells (Marcotti et al. 1999). Besides I_{K1}, hair cell-like cells also showed a small outward I_K and a sustained inward Ca^{2+} current. This correlates with the expression of the Cav1.3 subunit by cells under 'hair cell conditions', confirmed by RT-PCR. In summary, these cells are an excellent system to study human ear differentiation and, as a biotechnological tool, allowed to define the conditions needed to induce differentiation into neurons and hair cells.

31.7 Critical Bottle Necks in the Large Scale Production of Clinical Grade Cells

31.7.1 Cell Isolation and Purity

The translation of any experimental stem cell approach into a real clinical therapy requires the development of standards and quality control (QC) processes that are suited to a very strict scrutiny by the regulatory bodies. Clinical production of cells will need to adhere to good manufacturing practices (GMP) to insure the delivery of a "cell drug" that is safe, reproducible and efficient. For this, all parts of the process would have to be optimized and well defined. To achieve this final goal, is necessary to develop the tools that will facilitate the scaling up of a controlled production process.

An early, important element to address is how to purify the relevant cells in an efficient and non-invasive manner. To prospectively isolate cells from complex tissues or mixtures, being these the cochlea or a mix population induced from pluripotent cells, we need suitable markers. An ideal approach would be to use cell surface markers to allow their purification by Fluorescent Automated Cell Sorting (FACS). Other fields, such as haematology, have developed quickly and haematopoetic stem cells advanced into clinical application because of the availability of well-defined surface markers. These have made possible the specific isolation and purification of progenitors for different lineages and the monitoring of their differentiation (Wognum et al. 2003). In the auditory field, we completely lack these

tools. The use of cell-specific regulatory elements driving reporter proteins such as GFP has been applied to the successful isolation of supporting cells from the postnatal mouse cochlea (White et al. 2006). Although highly useful for research in animal models, this approach requires genetic modifications of the target cell (or the generation of transgenic mutants in the case of animal models) that makes it either undesirable or totally unfeasible for clinical applications in humans. The elucidation of the transcriptome of the human auditory stem cells should yield strong candidates of surface molecules to screen with antibodies, as is has been done with glial precursors (Campanelli et al. 2008). This targeted, informed approach should be more advantageous than the random screening of available antibodies.

A potentially useful strategy for their purification could be the isolation, by flow cytometry, of 'side populations' based on the ability of certain stem cell-like cells to exclude Hoechst dye. This method was successfully used by Savary et al. (2007) to isolate a population of supporting cells from the mouse cochlea that retained progenitor properties. It is important to note that hFASCs express the ABCG2 transporter, which is believed to be the molecule responsible for the 'side population' phenotype. However, because is a functional assay it is often very difficult to standardize and different laboratories have obtained very dissimilar results while working in other systems (Sales-Pardo et al. 2006). In an effort to address this lack of markers, Hertzano et al. (2010) identified a cohort of 107 'cluster of differentiation (CD) antigens' expressed in the postnatal mouse inner ear. From these, CD44 was detected as a reliable marker for the outer pillar cells. In a follow-up study, they describe that CD326 stains sensory and non-sensory epithelial cells at day P0, while CD49f is specific to sensory epithelial cells (Hertzano et al. 2011).

31.7.2 Cell Expansion

31.7.2.1 Signalling Pathways That Control Stemness, Cell Growth and Viability

Another relevant feature to establish in the progression towards a clinical therapy is the identification of signalling cascades that would control proliferation, survival and maintenance of the undifferentiated phenotype. Interrogating gene array data could highlight potential pathways that can then be targeted with growth factors or small chemical compounds. For instance, the role of PI3K/AKT, MAPK/ERK and NFκß signalling in the preservation of human embryonic stem cell pluripotency and viability was detected by transcriptional profiling (Armstrong et al. 2006), while a similar approach has shown the importance of PDGF, TGF-beta, and FGF signalling for the growth of mesenchymal stem cells (Ng et al. 2008). Identifying important signalling pathways that control cell growth, stemness and viability will help in the design of improved culture media that will facilitate their expansion.

It is important to highlight the relevance of studying these events in the right experimental system. Although a lot of information obtained from animal cells has

proved to translate well to similar populations of human origin, relevant important differences are found on the behaviour of stem cells, primarily those regarding self-renewal of mouse and human ES cells (Sato et al. 2003; Ginis et al. 2004). Some of these differences have been attributed to a problem of 'timing' and the fact that blastocyst-derived human ES cells will 'drift', once in vitro, to become comparable to the more developed, epiblast stem cells derived from postimplantation mouse embryos. However, this model of 'cell progression' would still reflect an intrinsic, specie-related difference between blastocyt-derived ES cells in their ability to remain pluripotent in vitro (Brons et al. 2007; Tesar et al. 2007; Vallier et al. 2009).

On the other hand, information gathered from hFASCs is likely to be applicable to the production of auditory cells from other human sources such as ES or iPS cells.

31.7.2.2 The Genetic Signature of Auditory Stem Cells

The global analysis of gene expression, by using oligonucleotide arrays, is an extremely powerful tool to define the molecular identity of a cell population. A few years ago, it was initially applied to different populations of stem cells aiming to identify a set core of genes that will define 'stemness' across very different types of cells such as embryonic, neural and hematopoietic stem cells from adult and fetal origin (Ivanova et al. 2002; Ramalho-Santos et al. 2002). These studies were then criticized as producing lists of genes that were too broad and vague (Evsikov and Solter 2003; Fortunel et al. 2003), and not reflecting a true core of stem cell genes and the elusive 'stemness'. However, although this criticism was partially appropriate, these experiments were successful in identifying nanog, a gene that was later independently characterized by two different laboratories as having a pivotal role in stem cell behaviour (Chambers et al. 2003; Mitsui et al. 2003). Since then gene arrays have been extensively applied to study stem cell populations, and the results have been most useful when the experimental systems are well characterized and the data interrogated by experiment-lead questions. When properly applied they have, for instance, allowed the identification of signalling cascades involved in the survival and differentiation of human embryonic stem cells (Enver et al. 2005), and more recently, the identification of regulatory networks that define different classes of stem cells (Muller et al. 2008).

In the ear, gene arrays have been used to study the developing cochlea (Sajan et al. 2007), the organ after injury (Hawkins et al. 2003, 2007) and different immortalized mouse cell lines while proliferating and differentiating (Rivolta et al. 2002; Lawoko-Kerali et al. 2004). Their comprehensive use in hearing research as been recently reviewed in Rivolta and Holley (2008).

31.7.2.3 Screening of Small Compound Libraries

Small compound libraries currently available contain hundred of thousands or even millions of chemicals, targeting different molecules and signalling pathways. The

complexity of these huge, highly diverse libraries created through combinatorial chemistry sometimes conspires against the applicability in the search for a specific phenotype (reviewed in Emre et al. 2007). It is possible then to select a more specific approach, where compounds are synthesized based on key biological motifs used as 'core' scaffolds, and targeting particular sets of molecules or protein families (i.e. kinases, phosphatases, etc. (Ding et al. 2002)). This target-based approach is feasible when some prior knowledge allows the pre-selection of 'candidate' signalling cascades that are likely to be relevant for the interested phenotype. On the other hand, in a phenotype-based approach, high throughput screening of unbiased chemical libraries could lead to the identification of new pathways. For instance, in Chen et al. (2006), a phenotype-based screening identified pluripotin as a promoter of self renewal in mES cells. Characterization of this compound identified it as an inhibitor of Erk1 and RasGAP, and that the inhibition of these two targets was necessary for the pluripotent phenotype.

Oligonucleotide microarrays and screening of small compound libraries are not the only high throughput screening methods that could produce vital results from auditory stem cells. Combinatorial evaluation of synthetic biomaterials uses a library of photopolymerizable material arranged in a microarray format. Using this approach, interaction between cells and the physical surfaces in which they are grown can be studied. In a particular study, the effect of more than 1,700 polymers on hESC growth and differentiation was explored (Anderson et al. 2004). This method could be particularly attractive to explore potential interactions between stem cells and electrodes, developing their interface and applicability in combination with cochlear implants. Other high-throughput screening methods applicable to stem cell biology are reviewed in Mei et al. (2007).

31.8 Cell Delivery

The routes for surgical delivery are another major factor for consideration when transplanting cells in the very small and delicate cochlear tissue. The main objective for transplantation is to deliver the cells into the target, damaged area. This will obviously depend on which type of degeneration has occurred and whether the primary aim is to replace HCs and/or SGNs (Fig. 31.2). Another challenge is to distribute the transplanted cells throughout the length of cochlea while minimizing further damage from the transplantation. A few articles have been published showing different cell delivery techniques into mammalian cochleae, which are summarized in the following section.

31.8.1 Intra-perilymphatic Transplantation

Perilymphatic transplantation via the scala tympani is probably the most used delivery technique so far. Positive features are the relative bigger volume of the space

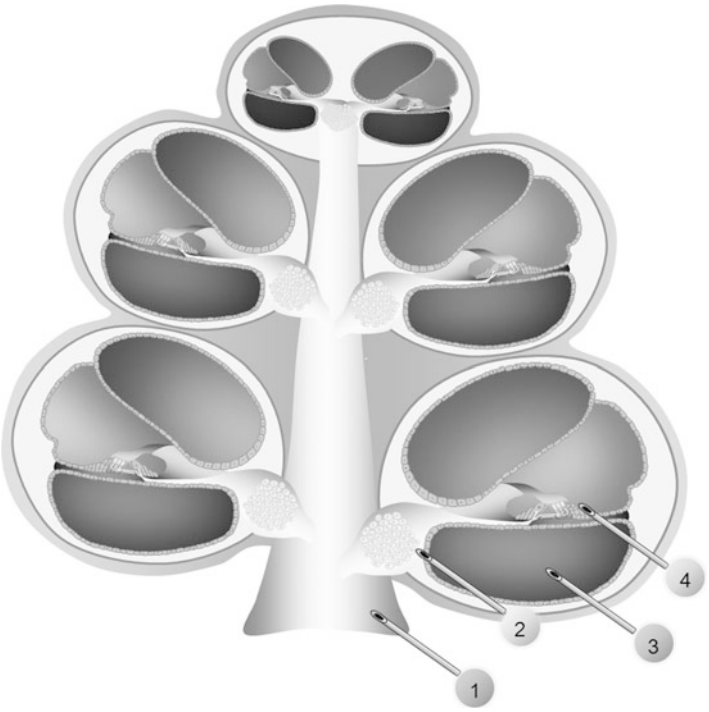


Fig. 31.2 Schematic illustration showing the routes for cell delivery into the cochlea. For neuronal replacement, cells can be delivered into the cochlear nerve trunk (*1*) or directly into the Rosenthal's canal in the modiolus (*2*). Alternatively, cells can be injected into the scala tympani (*3*) or, in order to target the organ of Corti, directly into the scala media (*4*)

when compared to the other cochlear compartments together with the fact that its fluid runs along the entire cochlear length, making it the best vehicle to distribute transplanted cells throughout the cochlea. Moreover, surgical access to the perilymphatic duct is believed to cause less trauma to the cochlea, and it can be done either through a cochleostomy in close proximity to the round window or through the round window itself. Access to the perilymphatic compartment is not just limited to the scala tympanic in the basal turn. Iguchi et al. (2004) have shown that it is possible to reach it via the lateral semicircular canal (LSCC). Transplanted neuropheres derived from mESCs were found in every cochlear turn in all experimental animals, with a larger ratio of cells found in the scala vestibuli than in the scala tympani. A small number of cells were also found in the scala media (around 0.4%). This experiment suggested that this surgical technique would cause only a minimal ABR threshold shift (~10–15 dB SPL) in the high frequency response region (40 kHz) (Iguchi et al. 2004). There was no evidence of cells being able to reach the modiolus by this approach. Even though the number of cells in the scala media was modest, it would suggest that they could migrate into the endolymphatic compartment from the perilymphatic region. How could cells manage to cross into a tightly sealed

compartment and survive in an environment with high potassium concentration, remains to be established. However, several other transplantation experiments using mESCs and MSCs delivered into the scala tympani found cells localized only to the perilymphatic space (Coleman et al. 2006; Matsuoka et al. 2006, 2007). On a different study, using neural stem cells transplanted into the scala tympani, cells were able to migrate to an area very close to the Rosenthal's canal (Hu et al. 2005b). However, the number of surviving cells was very low, and differentiating cells were only obtained when transduced with neurogenin2. Supplementation with GDNF has also been found to promote survival and migration of transplanted cells into the cochlear modiolus (Altschuler et al. 2008). Dorsal root ganglion neurons co-grafted with mESCs would appear to promote migration and survival of the undifferentiated cells. In this experiment, mESCs were found to migrate into the area of SGN via the intrascalar access, suggesting that DRG co-graft not only release growth factors to support the survival but also provide a structural matrix for cell growth in the fluid-filled compartment (Hu et al. 2005a). Cells transplanted in the scala tympani are thought to migrate to the modiolus via holes located in the internal bony wall, known as '*canaliculae perforantes*'. This area is highly porous, and may provide windows big enough for transplanted cells to migrate into the Rosenthal's canal. However, the size of transplanted cells would be a major factor limiting cell migration through these bony pores (Sekiya et al. 2007). A report by Parker et al. (2007) described general distribution of cells throughout different cochlear compartments when using a perfusion system (Parker et al. 2007). Since the initial scala tympani delivery produced a localized lump of cells, they explored perfusion by using a syringe pump. As described above, this group used Y-FISH technique to trace the transplanted cells and showed migration to different areas in the cochlea including the organ of Corti, Rosenthal canal and even to the spiral ligament. Transplanted cells would appeared to have differentiated into both hair cells, supporting cells (pillar, Deiter's, phalangeal), SGN, satellite cell and cells in the spiral ligament, although cell fusion could not be categorically ruled out.

31.8.2 Transplantation Aiming to the Organ of Corti via the Scala Media

There have been very few reports aiming to introduce stem cells directly into the scala media, and it remains the most technically difficult. The main reason for this approach would be to target the replacement of HCs, however several biological barriers would need to be overcome. The endolymph, the fluid located in this compartment, contains high levels of potassium (~150 mM). These concentrations are toxic to many cell types, and it could lead to a very limited viability of transplanted cells. Moreover, the complex cytoarchitecture of the organ of Corti represents another challenge. The epithelium is a tightly sealed barrier and it will be difficult for the transplanted cells to break through the adherent and tight junctions between hair cells and supporting cells in order to home and graft. An initial attempt into

scala media transplantation was described in mice, by delivering cells through the cochlear lateral wall (CLW) of the second cochlear turn. This approach revealed the distribution of transplanted cells in all three cochlear chambers, that is scala media, scala vestibuli and scala tympani, with a relative distribution of cells of 62.1, 20.8 and 17.1% respectively. There were no reports of differentiation and integration into the host tissue, neither confirmation of survival of transplanted cells in the endolymph. To compound the problem, the surgical intervention produced a significant elevation of ABR thresholds over all frequency ranges after 3 days post- operation and there was no significant recover of threshold observed at any frequency (Iguchi et al. 2004). The access via the CLW obviously damaged cochlear function, specially the structures needed to maintain homeostasis of K^+ and/or endocochlear potential. It has been shown that by this lateral approach to the scala media is possible to damage the stria vascularis and cochlear blood supply (Izumikawa et al. 2005). A different surgical access to the scala media was then developed by approaching through the basilar membrane with a cannula via the cochlear round window. This technique was established by Hildebrand et al. (2005) to deliver neuroectoderm-like embryoid bodies to deafened guinea pigs. There was no evidence of damage to the organ of Corti or Reissner's membrane attributable to the surgical procedure in any animal after surgical delivery. This evidence was confirmed by retaining the ABR threshold level after surgery when compared to the control group. The transplanted cells were found in all three cochlear chambers, scala media, scala tympani and scala vestibuli. The overall survival rate of transplanted cell was around 19.1%, 9 weeks after transplantation. Around 14% of cells survived in the scala media, and some were localized close to the damaged organ of Corti; however, there was no evidence of extensive cell differentiation and integration into the host tissue (Hildebrand et al. 2005). This would indicate that some cells are able to survive, probably after partial differentiation, in a potassium-rich environment. However, conclusive evidence for the integration of transplanted cells into the damaged organ of Corti is still missing.

31.8.3 Transplantation via the Modiolus and into the Cochlear Nerve Trunk

The modiolar and cochlear nerve trunk route for transplantation are mostly aimed to replace the degeneration of SGNs. Because of the difficulties described above, hair cell replacement is still a long way away. However, targeting sensory neurons appears as a far more realistic application in the short term. Moreover, a cell based-therapy to reconstitute the nerve cells could be implemented in combination with the currently available cochlear implants. As mentioned before, these devices can substitute hair cell function but still require the existence of SGNs to function, as the bridge of signals to the central nervous system. Secondary degeneration of SGNs after hair cell loss is hugely problematic and this severely affects the chances of deaf patients to receive cochlear implantation. For these reasons, some research groups

have turned their interest to study the regeneration of SGNs and transplantation via cochlear nerve seem to be the most reasonable approach to deliver stem cells to the target location in Rosenthal's canal. Interesting results from mESCs transplantation via cochlear nerve trunk access has shown both of peripheral and central migration along the cochlear nerve from the injection site. Transplanted cells were found close to the ventral cochlear nucleus (VCN) but the number of cells that migrated to Rosenthal's canal is still limited (Hu et al. 2004). Similar results were obtained when rat E13 embryonic SGNs progenitors were used. The transplanted cells grafted in the nerve trunk between the brain stem and the internal auditory meatus (IAM), but cells could not migrate further that the CNS/PNS transition boundary and failed to reach the cochlea (Palmgren et al. 2011). There is evidence of cells undergoing differentiation to SGN-like morphology after transplanted via the IAM of the intact cochlear nerve (Sekiya et al. 2007). Mouse conditionally immortalised neuroblasts (VOT-N33) were used in this study and distributed in the cochlear modiolus in different levels. Surprisingly, the transplanted cells via the IAM approach differentiated into a bipolar morphology and showed a very strong neuronal marker staining of β-tubulin III just 7 days after transplantation. Exploring a similar route, mESC-derived neuroprogenitor cells were implanted into the cochlear nerve trunk of deafened animal model. The surgical approach was made at the bony area that separates the cochlear nerve from the floor of round window niche. This study has shown very nice engraftment of transplanted cell into the host tissue. Even though, very small number of cells showed migration to the Rosenthal's canal but instead formed ectopic ganglions at the transplantation site with projections into the Rosenthal's canal and believed to reach to the organ of Corti (Corrales et al. 2006). Although the cochlear nerve trunk approach seems to allow for a more targeted delivery of cells, it still unable to introduce the transplanted cells into their ultimate destination, the Rosenthal canal. The osseous spiral lamina, which surrounds the SGNs might be a major barrier for cell migration. However, a few studies have shown fibres that project from ectopic, transplanted ganglion and penetrate into the Rosenthal canal. These are assumed to make synaptic connections at the base of hair cells. More evidence is still needed to confirm that this kind of synaptic connection is sufficient for functional recovery, together with the establishment of connections at the cochlear nucleus. Functional hearing measurements are also required to determine the level of mechanical damage from the surgical techniques that may further deteriorate residual auditory nerve.

Two studies have specifically compared these different delivery routes using the same cell type. The first one, produced by Lang et al., explored the perilymphatic approach via scala tympani, the scala media and the Rosenthal's canal approach through the round window niche using neuralized mESCs (Lang et al. 2008). Two weeks after transplantation, cell survival into the endolymphatic compartment was very low when compared to the perilymphatic injection. Most of the transplanted cells were dead or dying, showing evidence of apoptosis. The Rosenthal's canal approach performed the best, with large numbers of surviving transplanted cells labelling with neuron and glial markers. The microenvironment in the Rosenthal's canal might be vital to provide the appropriate niche for the

transplanted cell. The results of this study are highly encouraging for the delivery into the Rosenthal's canal area, but there was no evidence about migration beyond the transplantation site.

The other study aimed to compare amongst a cochleostomy into the scala tympani, the auditory nerve approach via translabyrinth and a direct access to the Rosenthal's canal through the osseous spiral lamina wall of the scala tympani (Backhouse et al. 2008). The transplantation was done not with cells, but with biocompatible microspheres delivered with or without the hydrogel, the matrix to minimize dispersal. Endogenous SGNs survival was measured, and the generation of areolar fibrous tissue and bone formation were counted to indicate inflammatory tissue response. The translabyrinthine approach produced the largest inflammatory responses and also damaged the SGNs. Hydrogel was claimed to be an effective biocompatible matrix, which could potentially retain microspheres at their implant site and caused no reaction or inflammatory response affecting the survival of SGNs.

31.9 Models to Study Cell Transplantation in the Inner Ear

Several factors are critical for the success of a transplantation project. These include, for instance, the survival and homing of cells into the host tissues, the migration of transplanted cells to the target site, the differentiation of cells into appropriate phenotypes and their ability to regenerate the functional connections. All these variables are the result of a balanced interaction between the intrinsic potential of the donor cells and the properties provided by the microenvironment of the host tissue. Therefore, the models used for transplantation experiments should be considered carefully. In the different models, deafness can be induced in a targeted manner, hitting primarily the hair cells, the spiral ganglion neurons or the fibrocytes of the lateral wall. Various kinds of chemical substances have been used, showing different cellular targets. Aminoglycosides antibiotics have been widely used to damage primarily hair cells, subsequently causing SNHL. A secondary degeneration of SGNs takes place later (Coleman et al. 2006). The glycoside and Na + K + ATPase inhibitor, ouabain, has been broadly employed to specifically damage SGNs. Outer hair cells and stria vascularis are not affected, as shown by histology and the preservation of distorted product oto acoustic emissions (DPOAE) and endocochlear potential (EP), respectively (Schmiedt et al. 2002). Ouabain application via the cochlear round window niche can increase the threshold of cochlear compound action potential (CAP) in just only 3 h after application and SGNs undergo apoptosis after 12 h. This drug targets specifically type I spiral ganglion neurons (Lang et al. 2005). Because of this specificity, the ouabain application has been used as a model of neuropathic deafness in several transplantation studies (Corrales et al. 2006; Shi et al. 2007; Lang et al. 2008). A comparative study between transplantation in the intact cochlea versus a model of hair cell injury by neomycin, revealed that the survival rate of transplanted cells was better in the neomycin-treated animals.

Moreover, the differentiation potential of transplanted cells was also greater in the deafened model (Hu et al. 2005a). This result suggests that the microenvironment provided by damaged host tissue is a significant factor for viability and differentiation of transplanted cells. The length of timing after injury was also found to have a major impact in the survival of implanted cells. In an early post-injury transplantation, made 1–3 days after the deafening protocol, cell survival was substantially better than when transplantation was done 7 days after induction of deafness (Lang et al. 2008). Finally, noise-induced deafness has also been employed as a model for transplantation. Exposure to loud noise (112 dB level) for 72 h can induce deafness in guinea pigs and generate damage to different cell types including cells in stria vascularis, hair cells and supporting cells of the organ of Corti. The area of damage corresponded with frequency of sound stimulus (Parker et al. 2007).

31.10 Functional Tests to Explore Recovery of Hearing After Transplantation

To complete the analysis of a transplantation study, it is necessary to monitor for functional recovery. Many types of hearing measurements have been conducted to evaluate the level of deafness, establishing the severity of injury and detecting recovery after transplantation. Otoacoustic emissions are sounds produced as a consequence of electromechanical feedback from the outer hair cells (OHC). Distortion product otoacoustic emissions (DPOAEs) are a measure of the sensitivity of the cochlea. Distortion products are generated when two tones with frequencies F1 and F2 are mixed in a nonlinear amplifier. In the cochlea, the amplifier is the OHCs and a principle distortion product is 2 F1 –F2. The magnitude of the distortion products generated by the cochlea is determined by the level of the primary tones and also by their ratio, which for the mouse cochlea is about 1.24. By varying the frequency and levels of the primary tones it is possible to derive an audiogram and also to derive input-output functions for each frequency region of the cochlea. DPOAEs can therefore be used to check the functional status of OHC in a given region of the cochlea (Kemp 2002). Some experiments had obtained DPOAE measurements to evaluate the degree of deafness after drugs application and/or the level of cochlea perturbation after transplantation (Corrales et al. 2006; Lang et al. 2008). Compound action potential recording is the measurement that related to both HCs and SGNs function. This technique can give sensitive inference of cochlear function since the recording electrode is placed at the cochlear round window and it can detect the evoked responses from sound stimulation at different frequencies. Evoked auditory brainstem responses (ABRs) work in a similar way, but the recording is done from distant, surface electrodes. It measures the discharge of auditory neurons in response to a given tone and the subsequent series of potentials generated by the nuclei of the central auditory pathway. It is recorded as a pattern of a series of waves that represent different aspects of the pathway. Because of this, it is an ideal tool to study the integration of grafted cells into host tissues and the reconnection of the cochlear

nerve to auditory brainstem nuclei. ABRs have been used in transplantation studies to verify the level of injury after surgery (Iguchi et al. 2004; Bogaerts et al. 2008), to determine deafness after drug application (Coleman et al. 2006) and to ascertain recovery after transplantation (Ito et al. 2001; Sekiya et al. 2007). There is a correlation between the three main parameters measured in ABRs (amplitude of the signal, threshold and latency of the response) and their anatomical significance. The amplitude of the first wave reflects primarily the sum of neural firings of a large population of SGNs, whereas threshold level represents the function of only the most sensitive auditory nerve fibres (Agterberg et al. 2009). An increment of latency after induction of deafness correlates well with the degeneration of dendrites and a reduction of the number of myelin layers in the fibers. In summary, the use of a single test or a combination of them should be decided depending which part of the auditory pathway the cell transplantation experiment is aiming to explore.

31.11 Conclusions and Future Perspectives

Although a substantial volume of information is starting to mount about this potentially revolutionary technique, a lot more research is still needed to establish the ideal conditions for the system to work. A correct balance between the right intrinsic factors (such as the donor cell type) and extrinsic factors (such as the host environment and means of delivery), still needs to be achieved. Undifferentiated ESCs have shown a good migratory capacity but less ability to differentiate into the target cell types. The neuroprogenitor cells appear to differentiate into SGNs but seem to stay mostly at the transplanted site. Systems that allow the control of directed differentiation, such as the neurog1 inducible one (Reyes et al. 2008), are elegant solutions to establish proof of principle but, since they involve substantial genetic modifications of the donor cell, are unlikely to be of clinical application. Studies with model cell systems of murine origin are highly valuable. However, given the important differences encountered between species (primarily in the stem cell biology field) more studies with human cell types are required to establish conditions of clinical relevance. Since the use of factors such as NTFs supplements seems to promote survival and differentiation, the combination of cell transplantation together with NTFs supplementation should be worthwhile to explore. Along similar lines, studies combining the potential of stem cells and cochlear implants are critically needed. Regarding the transplantation route, the cochlear modiolar approach to gain direct access to the Rosenthal's canal appears to be the best system for SGN replacement. However, for hair cell replacement, the delivery techniques into the scala media are still rudimentary and produce a substantial amount of damage. Further development and refinement is clearly needed. Finally, promoting transdifferentiation of supporting cells into hair cells can be considered cell replacement, albeit from an endogenous source. Alteration of the supporting cell phenotype, by removing the cell cycle inhibition imposed by $p27^{Kip1}$ or by promoting the expression of the

hair cell transcription factor *Math1/Atoh1* (Kawamoto et al. 2003; Izumikawa et al. 2005) could have great potential for hair cell generation *in vivo*.

Acknowledgements This work was supported by grants from the Medical Research Council (MRC) and Action on Hearing Loss (AoHL) to MR.

References

Agterberg MJ, Versnel H, van Dijk LM, de Groot JC, Klis SF (2009) Enhanced survival of spiral ganglion cells after cessation of treatment with brain-derived neurotrophic factor in deafened guinea pigs. J Assoc Res Otolaryngol 10:355–367

Altschuler RA, O'Shea KS, Miller JM (2008) Stem cell transplantation for auditory nerve replacement. Hear Res 242:110–116

Anderson DG, Levenberg S, Langer R (2004) Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. Nat Biotechnol 22:863–866

Armstrong L, Hughes O, Yung S, Hyslop L, Stewart R, Wappler I, Peters H, Walter T, Stojkovic P, Evans J, Stojkovic M, Lako M (2006) The role of PI3K/AKT, MAPK/ERK and NFkappabeta signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis. Hum Mol Genet 15:1894–1913

Backhouse S, Coleman B, Shepherd R (2008) Surgical access to the mammalian cochlea for cell-based therapies. Exp Neurol 214:193–200

Balasubramaniyan V, de Haas AH, Bakels R, Koper A, Boddeke HW, Copray JC (2004) Functionally deficient neuronal differentiation of mouse embryonic neural stem cells in vitro. Neurosci Res 49:261–265

Bogaerts S, Douglas S, Corlette T, Pau H, Saunders D, McKay S, Oleskevich S (2008) Microsurgical access for cell injection into the mammalian cochlea. J Neurosci Methods 168:156–163

Brons IG, Smithers LE, Trotter MW, Rugg-Gunn P, Sun B, de Sousa C, Lopes SM, Howlett SK, Clarkson A, Ahrlund-Richter L, Pedersen RA, Vallier L (2007) Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448:191–195

Campanelli JT, Sandrock RW, Wheatley W, Xue H, Zheng J, Liang F, Chesnut JD, Zhan M, Rao MS, Liu Y (2008) Expression profiling of human glial precursors. BMC Dev Biol 8:102

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113:643–655

Chen P, Segil N (1999) p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. Development 126:1581–1590

Chen S, Do JT, Zhang Q, Yao S, Yan F, Peters EC, Scholer HR, Schultz PG, Ding S (2006) Self-renewal of embryonic stem cells by a small molecule. Proc Natl Acad Sci USA 103:17266–17271

Chen W, Cacciabue-Rivolta DI, Moore HD, Rivolta MN (2007) The human fetal cochlea can be a source for auditory progenitors/stem cells isolation. Hear Res 233:23–29

Chen W, Johnson SL, Marcotti W, Andrews PW, Moore HD, Rivolta MN (2009a) Human fetal auditory stem cells can be expanded in vitro and differentiate into functional auditory neurons and hair cell-like cells. Stem Cells 27:1196–1204

Chen WJN, Jonhson SL, Kuhn S, Marcotti W, Andrews PW, Moore HD, Rivolta MN (2009) FGF signaling can induce the generation of cochlear progenitors from hESCs with the potential to differentiate into functional hair cell-like cells and neurons UK National Stem Cell Network. Oxford, UK, p 99

Chen W, Jongkamonwiwat N, Abbas L, Eshtan SJ, Johnson SL, Kuhn S, Milo M, Thurlow JK, Andrews PW, Marcotti W, Moore HD, Rivolta MN (2012) Restoration of auditory evoked responses by human ES-cell-derived otic progenitors. Nature 490(7419):278–282. doi:10.1038/nature11415

Coleman B, Hardman J, Coco A, Epp S, de Silva M, Crook J, Shepherd R (2006) Fate of embryonic stem cells transplanted into the deafened mammalian cochlea. Cell Transplant 15:369–380

Coleman B, Fallon JB, Pettingill LN, de Silva MG, Shepherd RK (2007) Auditory hair cell explant co-cultures promote the differentiation of stem cells into bipolar neurons. Exp Cell Res 313:232–243

Corrales CE, Pan L, Li H, Liberman MC, Heller S, Edge AS (2006) Engraftment and differentiation of embryonic stem cell-derived neural progenitor cells in the cochlear nerve trunk: growth of processes into the organ of Corti. J Neurobiol 66:1489–1500

Daudet N, Vago P, Ripoll C, Humbert G, Pujol R, Lenoir M (1998) Characterization of atypical cells in the juvenile rat organ of corti after aminoglycoside ototoxicity. J Comp Neurol 401:145–162

Ding S, Gray NS, Wu X, Ding Q, Schultz PG (2002) A combinatorial scaffold approach toward kinase-directed heterocycle libraries. J Am Chem Soc 124:1594–1596

Duan M, Agerman K, Ernfors P, Canlon B (2000) Complementary roles of neurotrophin 3 and a N-methyl-D-aspartate antagonist in the protection of noise and aminoglycoside-induced ototoxicity. Proc Natl Acad Sci USA 97:7597–7602

Duan M, Venail F, Spencer N, Mezzina M (2004) Treatment of peripheral sensorineural hearing loss: gene therapy. Gene Ther 11(Suppl 1):S51–S56

Eisen MD (2003) Djourno, Eyries, and the first implanted electrical neural stimulator to restore hearing. Otol Neurotol 24:500–506

Emre N, Coleman R, Ding S (2007) A chemical approach to stem cell biology. Curr Opin Chem Biol 11:252–258

Enver T, Soneji S, Joshi C, Brown J, Iborra F, Orntoft T, Thykjaer T, Maltby E, Smith K, Dawud RA, Jones M, Matin M, Gokhale P, Draper J, Andrews PW (2005) Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. Hum Mol Genet 14:3129–3140

Ernfors P, Van De Water T, Loring J, Jaenisch R (1995) Complementary roles of BDNF and NT-3 in vestibular and auditory development. Neuron 14:1153–1164

Ernfors P, Duan ML, ElShamy WM, Canlon B (1996) Protection of auditory neurons from aminoglycoside toxicity by neurotrophin-3. Nat Med 2:463–467

Evsikov AV, Solter D (2003) Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". Science 302:393, author reply 393

Fekete DM, Muthukumar S, Karagogeos D (1998) Hair cells and supporting cells share a common progenitor in the avian inner ear. J Neurosci 18:7811–7821

Forge A, Li L, Corwin JT, Nevill G (1993) Ultrastructural evidence for hair cell regeneration in the mammalian inner ear. Science 259:1616–1619

Forge A, Li L, Nevill G (1998) Hair cell recovery in the vestibular sensory epithelia of mature guinea pigs. J Comp Neurol 397:69–88

Fortunel NO, Otu HH, Ng HH, Chen J, Mu X, Chevassut T, Li X, Joseph M, Bailey C, Hatzfeld JA, Hatzfeld A, Usta F, Vega VB, Long PM, Libermann TA, Lim B (2003) Comment on "'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". Science 302:393, author reply 393

Fritzsch B, Farinas I, Reichardt LF (1997) Lack of neurotrophin 3 causes losses of both classes of spiral ganglion neurons in the cochlea in a region-specific fashion. J Neurosci 17:6213–6225

Fu Y, Wang S, Liu Y, Wang J, Wang G, Chen Q, Gong S (2009) Study on neural stem cell transplantation into natural rat cochlea via round window. Am J Otolaryngol 30:8–16

Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, Amit M, Hoke A, Carpenter MK, Itskovitz-Eldor J, Rao MS (2004) Differences between human and mouse embryonic stem cells. Dev Biol 269:360–380

Hardie NA, Shepherd RK (1999) Sensorineural hearing loss during development: morphological and physiological response of the cochlea and auditory brainstem. Hear Res 128:147–165

Havenith S, Versnel H, Agterberg MJ, de Groot JC, Sedee RJ, Grolman W, Klis SF (2011) Spiral ganglion cell survival after round window membrane application of brain-derived neurotrophic factor using gelfoam as carrier. Hear Res 272:168–177

Hawkins RD, Bashiardes S, Helms CA, Hu L, Saccone NL, Warchol ME, Lovett M (2003) Gene expression differences in quiescent versus regenerating hair cells of avian sensory epithelia: implications for human hearing and balance disorders. Hum Mol Genet 12:1261–1272

Hawkins RD, Bashiardes S, Powder KE, Sajan SA, Bhonagiri V, Alvarado DM, Speck J, Warchol ME, Lovett M (2007) Large scale gene expression profiles of regenerating inner ear sensory epithelia. PLoS One 2:e525

Hertzano R, Puligilla C, Chan SL, Timothy C, Depireux DA, Ahmed Z, Wolf J, Eisenman DJ, Friedman TB, Riazuddin S, Kelley MW, Strome SE (2010) CD44 is a marker for the outer pillar cells in the early postnatal mouse inner ear. J Assoc Res Otolaryngol 11:407–418

Hertzano R, Elkon R, Kurima K, Morrisson A, Chan SL, Sallin M, Biedlingmaier A, Darling DS, Griffith AJ, Eisenman DJ, Strome SE (2011) Cell type-specific transcriptome analysis reveals a major role for Zeb1 and miR-200b in mouse inner ear morphogenesis. PLoS Genet 7:e1002309

Hildebrand MS, Dahl HH, Hardman J, Coleman B, Shepherd RK, de Silva MG (2005) Survival of partially differentiated mouse embryonic stem cells in the scala media of the guinea pig cochlea. J Assoc Res Otolaryngol 6:341–354

Holley MC (2005) Keynote review: the auditory system, hearing loss and potential targets for drug development. Drug Discov Today 10:1269–1282

Hu Z, Ulfendahl M, Olivius NP (2004) Survival of neuronal tissue following xenograft implantation into the adult rat inner ear. Exp Neurol 185:7–14

Hu Z, Andang M, Ni D, Ulfendahl M (2005a) Neural cograft stimulates the survival and differentiation of embryonic stem cells in the adult mammalian auditory system. Brain Res 1051:137–144

Hu Z, Wei D, Johansson CB, Holmstrom N, Duan M, Frisen J, Ulfendahl M (2005b) Survival and neural differentiation of adult neural stem cells transplanted into the mature inner ear. Exp Cell Res 302:40–47

Hu Z, Ulfendahl M, Prieskorn DM, Olivius P, Miller JM (2009) Functional evaluation of a cell replacement therapy in the inner ear. Otol Neurotol 30:551–558

Iguchi F, Nakagawa T, Tateya I, Endo T, Kim TS, Dong Y, Kita T, Kojima K, Naito Y, Omori K, Ito J (2004) Surgical techniques for cell transplantation into the mouse cochlea. Acta Otolaryngol Suppl 551:43–47

Ito J, Murata M, Kawaguchi S (2001) Regeneration and recovery of the hearing function of the central auditory pathway by transplants of embryonic brain tissue in adult rats. Exp Neurol 169:30–35

Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, Lemischka IR (2002) A stem cell molecular signature. Science 298:601–604

Izumikawa M, Minoda R, Kawamoto K, Abrashkin KA, Swiderski DL, Dolan DF, Brough DE, Raphael Y (2005) Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. Nat Med 11:271–276

Jeon SJ, Fujioka M, Kim SC, Edge AS (2011) Notch signaling alters sensory or neuronal cell fate specification of inner ear stem cells. J Neurosci 31:8351–8358

Kada S, Nakagawa T, Ito J (2009) A mouse model for degeneration of the spiral ligament. J Assoc Res Otolaryngol 10:161–172

Kamiya K, Fujinami Y, Hoya N, Okamoto Y, Kouike H, Komatsuzaki R, Kusano R, Nakagawa S, Satoh H, Fujii M, Matsunaga T (2007) Mesenchymal stem cell transplantation accelerates hearing recovery through the repair of injured cochlear fibrocytes. Am J Pathol 171:214–226

Kawamoto K, Ishimoto S, Minoda R, Brough DE, Raphael Y (2003) Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. J Neurosci 23:4395–4400

Kawamoto K, Izumikawa M, Beyer LA, Atkin GM, Raphael Y (2009) Spontaneous hair cell regeneration in the mouse utricle following gentamicin ototoxicity. Hear Res 247:17–26

Kelley MW, Talreja DR, Corwin JT (1995) Replacement of hair cells after laser microbeam irradiation in cultured organs of corti from embryonic and neonatal mice. J Neurosci 15:3013–3026

Kemp DT (2002) Otoacoustic emissions, their origin in cochlear function, and use. Br Med Bull 63:223–241

Kim JB, Greber B, Arauzo-Bravo MJ, Meyer J, Park KI, Zaehres H, Scholer HR (2009) Direct reprogramming of human neural stem cells by OCT4. Nature 461(7264):649–653

Kujawa SG, Liberman MC (2006) Acceleration of age-related hearing loss by early noise exposure: evidence of a misspent youth. J Neurosci 26:2115–2123

Lang H, Schulte BA, Schmiedt RA (2005) Ouabain induces apoptotic cell death in type I spiral ganglion neurons, but not type II neurons. J Assoc Res Otolaryngol 6:63–74

Lang H, Schulte BA, Goddard JC, Hedrick M, Schulte JB, Wei L, Schmiedt RA (2008) Transplantation of mouse embryonic stem cells into the cochlea of an auditory-neuropathy animal model: effects of timing after injury. J Assoc Res Otolaryngol 9:225–240

Lawoko-Kerali G, Milo M, Davies D, Halsall A, Helyer R, Johnson CM, Rivolta MN, Tones MA, Holley MC (2004) Ventral otic cell lines as developmental models of auditory epithelial and neural precursors. Dev Dyn 231:801–814

Li H, Liu H, Heller S (2003a) Pluripotent stem cells from the adult mouse inner ear. Nat Med 9:1293–1299

Li H, Roblin G, Liu H, Heller S (2003b) Generation of hair cells by stepwise differentiation of embryonic stem cells. Proc Natl Acad Sci USA 100:13495–13500

Malgrange B, Thiry M, Van De Water TR, Nguyen L, Moonen G, Lefebvre PP (2002) Epithelial supporting cells can differentiate into outer hair cells and Deiters' cells in the cultured organ of Corti. Cell Mol Life Sci 59:1744–1757

Marcotti W, Geleoc GS, Lennan GW, Kros CJ (1999) Transient expression of an inwardly rectifying potassium conductance in developing inner and outer hair cells along the mouse cochlea. Pflugers Arch 439:113–122

Matsuoka AJ, Kondo T, Miyamoto RT, Hashino E (2006) In vivo and in vitro characterization of bone marrow-derived stem cells in the cochlea. Laryngoscope 116:1363–1367

Matsuoka AJ, Kondo T, Miyamoto RT, Hashino E (2007) Enhanced survival of bone-marrow-derived pluripotent stem cells in an animal model of auditory neuropathy. Laryngoscope 117:1629–1635

Mei Y, Goldberg M, Anderson D (2007) The development of high-throughput screening approaches for stem cell engineering. Curr Opin Chem Biol 11:388–393

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113:631–642

Moore DR, Shannon RV (2009) Beyond cochlear implants: awakening the deafened brain. Nat Neurosci 12:686–691

Morest DK, Cotanche DA (2004) Regeneration of the inner ear as a model of neural plasticity. J Neurosci Res 78:455–460

Muller FJ, Laurent LC, Kostka D, Ulitsky I, Williams R, Lu C, Park IH, Rao MS, Shamir R, Schwartz PH, Schmidt NO, Loring JF (2008) Regulatory networks define phenotypic classes of human stem cell lines. Nature 455:401–405

Nadol JB Jr (1997) Patterns of neural degeneration in the human cochlea and auditory nerve: implications for cochlear implantation. Otolaryngol Head Neck Surg 117:220–228

Nadol JB Jr, Young YS, Glynn RJ (1989) Survival of spiral ganglion cells in profound sensorineural hearing loss: implications for cochlear implantation. Ann Otol Rhinol Laryngol 98:411–416

Nayagam BA, Minter RL (2011) A comparison of in vitro treatments for directing stem cells toward a sensory neural fate. Am J Otolaryngol 33:37–46

Ng F, Boucher S, Koh S, Sastry KS, Chase L, Lakshmipathy U, Choong C, Yang Z, Vemuri MC, Rao MS, Tanavde V (2008) PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. Blood 112:295–307

Nishimura K, Nakagawa T, Ono K, Ogita H, Sakamoto T, Yamamoto N, Okita K, Yamanaka S, Ito J (2009) Transplantation of mouse induced pluripotent stem cells into the cochlea. Neuroreport 20:1250–1254

Oshima K, Grimm CM, Corrales CE, Senn P, Martinez Monedero R, Geleoc GS, Edge A, Holt JR, Heller S (2007) Differential distribution of stem cells in the auditory and vestibular organs of the inner ear. J Assoc Res Otolaryngol 8:18–31

Oshima K, Shin K, Diensthuber M, Peng AW, Ricci AJ, Heller S (2010) Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells. Cell 141:704–716

Palmgren B, Jin Z, Jiao Y, Kostyszyn B, Olivius P (2011) Horseradish peroxidase dye tracing and embryonic statoacoustic ganglion cell transplantation in the rat auditory nerve trunk. Brain Res 1377:41–49

Parker MA, Corliss DA, Gray B, Anderson JK, Bobbin RP, Snyder EY, Cotanche DA (2007) Neural stem cells injected into the sound-damaged cochlea migrate throughout the cochlea and express markers of hair cells, supporting cells, and spiral ganglion cells. Hear Res 232:29–43

Pettingill LN, Wise AK, Geaney MS, Shepherd RK (2011) Enhanced auditory neuron survival following cell-based BDNF treatment in the deaf guinea pig. PLoS One 6:e18733

Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA (2002) "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 298:597–600

Raphael Y (2002) Cochlear pathology, sensory cell death and regeneration. Br Med Bull 63:25–38

Rask-Andersen H, Bostrom M, Gerdin B, Kinnefors A, Nyberg G, Engstrand T, Miller JM, Lindholm D (2005) Regeneration of human auditory nerve. In vitro/in video demonstration of neural progenitor cells in adult human and guinea pig spiral ganglion. Hear Res 203:180–191

Reyes JH, O'Shea KS, Wys NL, Velkey JM, Prieskorn DM, Wesolowski K, Miller JM, Altschuler RA (2008) Glutamatergic neuronal differentiation of mouse embryonic stem cells after transient expression of neurogenin 1 and treatment with BDNF and GDNF: in vitro and in vivo studies. J Neurosci 28:12622–12631

Rivolta MN, Holley MC (2008) Gene arrays, cell lines, stem cells and sensory regeneration in mammalian ears. In: Salvi RJP, Popper AN, Fay RR (eds) Hair cell regeneration, repair and protection, vol 33. Springer, New York, pp 257–307

Rivolta MN, Halsall A, Johnson CM, Tones MA, Holley MC (2002) Transcript profiling of functionally related groups of genes during conditional differentiation of a mammalian cochlear hair cell line. Genome Res 12:1091–1099

Rubel EW, Dew LA, Roberson DW (1995) Mammalian vestibular hair cell regeneration. Science 267:701–707

Sajan SA, Warchol ME, Lovett M (2007) Toward a systems biology of mouse inner ear organogenesis: gene expression pathways, patterns and network analysis. Genetics 177:631–653

Sales-Pardo I, Avendano A, Martinez-Munoz V, Garcia-Escarp M, Celis R, Whittle P, Barquinero J, Domingo JC, Marin P, Petriz J (2006) Flow cytometry of the side population: tips & tricks. Cell Oncol 28:37–53

Sato N, Sanjuan IM, Heke M, Uchida M, Naef F, Brivanlou AH (2003) Molecular signature of human embryonic stem cells and its comparison with the mouse. Dev Biol 260:404–413

Savary E, Hugnot JP, Chassigneux Y, Travo C, Duperray C, Van De Water T, Zine A (2007) Distinct population of hair cell progenitors can be isolated from the postnatal mouse cochlea using side population analysis. Stem Cells 25:332–339

Schmiedt RA, Okamura HO, Lang H, Schulte BA (2002) Ouabain application to the round window of the gerbil cochlea: a model of auditory neuropathy and apoptosis. J Assoc Res Otolaryngol 3:223–233

Sekiya T, Yagihashi A, Shimamura N, Asano K, Suzuki S, Matsubara A, Namba A, Shinkawa H (2003) Apoptosis of auditory neurons following central process injury. Exp Neurol 184:648–658

Sekiya T, Kojima K, Matsumoto M, Kim TS, Tamura T, Ito J (2006) Cell transplantation to the auditory nerve and cochlear duct. Exp Neurol 198:12–24

Sekiya T, Holley MC, Kojima K, Matsumoto M, Helyer R, Ito J (2007) Transplantation of conditionally immortal auditory neuroblasts to the auditory nerve. Eur J Neurosci 25:2307–2318

Shi F, Corrales CE, Liberman MC, Edge AS (2007) BMP4 induction of sensory neurons from human embryonic stem cells and reinnervation of sensory epithelium. Eur J Neurosci 26:3016–3023

Shinohara T, Bredberg G, Ulfendahl M, Pyykko I, Olivius NP, Kaksonen R, Lindstrom B, Altschuler R, Miller JM (2002) Neurotrophic factor intervention restores auditory function in deafened animals. Proc Natl Acad Sci USA 99:1657–1660

Spahr AJ, Dorman MF (2004) Performance of subjects fit with the Advanced Bionics CII and Nucleus 3G cochlear implant devices. Arch Otolaryngol Head Neck Surg 130:624–628

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676

Tesar PJ, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL, Gardner RL, McKay RD (2007) New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448:196–199

Vallier L, Touboul T, Chng Z, Brimpari M, Hannan N, Millan E, Smithers LE, Trotter M, Rugg-Gunn P, Weber A, Pedersen RA (2009) Early cell fate decisions of human embryonic stem cells and mouse epiblast stem cells are controlled by the same signalling pathways. PLoS One 4:e6082

Webster DB (1992) Degeneration followed by partial regeneration of the organ of Corti in deafness (dn/dn) mice. Exp Neurol 115:27–31

Webster M, Webster DB (1981) Spiral ganglion neuron loss following organ of Corti loss: a quantitative study. Brain Res 212:17–30

White PM, Doetzlhofer A, Lee YS, Groves AK, Segil N (2006) Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. Nature 441:984–987

Wognum AW, Eaves AC, Thomas TE (2003) Identification and isolation of hematopoietic stem cells. Arch Med Res 34:461–475

Zhai S, Shi L, Wang BE, Zheng G, Song W, Hu Y, Gao WQ (2005) Isolation and culture of hair cell progenitors from postnatal rat cochleae. J Neurobiol 65:282–293

Chapter 32
Oral and Maxillo-facial

Kristina Arvidson, Michele Cottler-Fox, Sølve Hellem, and Kamal Mustafa

Abstract Regenerative medicine and dentistry are two rapidly growing fields of research with important clinical implications. Recent advances in cell biology, biotechnology, material science and tissue transplantation have been translated into new approaches to clinical repair and replacement of tissues and organs. In dentistry, a number of regenerative therapies and materials have been in clinical use for many years, to repair small and large defects involving multiple tissue types. Currently, various strategies are applied to stimulate healing of bone defects and to restore lost maxillofacial bone and periodontal support following traumatic insult, tumor ablation, disease or congenital deformities.

Bone tissue engineering is an emerging field using bone-forming cells seeded onto synthetic scaffolds to form hybrid constructs that can be used to regenerate tissues. There are numerous published case reports of the application of bone tissue engineering for oral and maxillofacial surgical reconstruction, periodontal tissue regeneration and sinus floor augmentation.

Mesenchymal stem cells (MSC) are currently the cells of choice for bone tissue engineering and can be isolated from many different tissues such as bone marrow, periosteum, and trabecular bone as well as from muscle, adipose tissue and synovial membrane. MSC have also been found among the cells derived from human

K. Arvidson, D.D.S., Ph.D. (✉)
Department of Clinical Dentistry – Centre for Clinical Dental Research,
University of Bergen, Årstadveien 17, POB 7800, 5020 Bergen, Norway

Vallmostigen 3, 17078 Solna, Stockholm, Sweden
e-mail: kristina.arvidson@iko.uib.no

S. Hellem, D.D.S., Ph.D.
Department of Clinical Dentistry – Oral and Maxillofacial Surgery,
University of Bergen, Årstadveien 17, POB 7800, 5020 Bergen, Norway

M. Cottler-Fox, M.D. • K. Mustafa, D.D.S., Ph.D.
Department of Clinical Dentistry – Centre for Clinical Dental Research,
University of Bergen, Årstadveien 17, POB 7800, 5020 Bergen, Norway

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_32,

umbilical cord: *in vivo*, these cells have demonstrated that they are capable of osteogenic differentiation, leading to bone formation and *in vitro* have shown adipogenic, chondrogenic, and osteogenic differentiation. Further, MSC have been identified in periodontal ligament, deciduous and permanent molar teeth. Recent research has shown that these cells have promising regenerative potential. Thus stem cell-based bone tissue engineering is a promising concept for reconstruction/regeneration of craniofacial defects but much work remains before this approach becomes a routine part of clinical practice.

32.1 Background

Modern dentistry is not limited to maintenance of dentition but has many subspecialties encompassing diagnosis and treatment of conditions affecting the oral and maxillofacial structures. In this relatively small area of the body, many different cells and tissue types occur in morphologically complex structures. Thus defects often involve multiple tissue types, including teeth and craniofacial bones, nerves and blood vessels, soft tissues such as mucosa, skin and muscles, salivary glands and specialized sensory organs.

The oral cavity plays an important role in daily living, including selection of nutritional intake through the complicated neural interactions of taste and smell. It is well documented in the scientific literature that teeth are important to both general health and quality of life through masticatory function, as well as to esthetics and speech. The oral cavity is important to general health and the quality of life because it is the initial organ of digestion: the first stage of the digestive process or mastication, the mechanical breaking up of solid food particles into smaller pieces by chewing and mixing them with saliva and its enzymes, occurs here. Natural dentition or a properly functioning substitute (fixed or removable prostheses) is of major importance to this function. The oral cavity is important to esthetics and speech because the physical appearance of the mouth, *i.e.* the teeth and lips, are essential to these functions and help in defining social and sexual attractiveness.

Over the past 50–60 years there have been major overall improvements in oral health, reflecting advances in research during this period. One of the most exciting developments is a change in traditional concepts of disease and its sequelae; from mechanical repair of damage to jaws and surrounding tissues, to a more biologically-based approach to treatment options. Advances in basic science using techniques from cellular and molecular biology have been translated into clinical practice. At the same time, clinical and epidemiological studies have improved methods of diagnosis, treatment and prevention of oral health problems.

A striking development is the decrease in the number of edentulous people over the past 40 years. The elderly are retaining their natural dentition and the mean number of standing teeth is higher than a generation ago. Improvements in periodontal health and oral health care are obvious. Many children are caries free or without active caries and the caries rate in adults has decreased. Important contributing

factors to caries prevention are water fluoridation and the widespread use of fluoride toothpaste, but it has also been shown that social, economic and geographic factors play important roles.

32.2 State of the Art

32.2.1 Loss of Permanent Teeth

One of the most common challenges for the dental clinician today, however, is rehabilitation following loss of the permanent teeth and the surrounding structures. Maintenance of good oral function is significant for general wellbeing, nutritional status and general health (Buhlin et al. 2002, 2003; Sheiham and Steele 2001; Nowjack-Raymer and Sheiham 2003). Loss of all the teeth or even of one tooth is a dramatic life event. For many people replacing missing teeth with complete dentures is unsatisfactory: not only are oral factors such as pain, taste perception and chewing capacity adversely affected, but the patient may also undergo marked psychological changes such as reduced self-image and loss of confidence in social situations (Trulsson et al. 2002).

Bone resorption is a common sequela to tooth extraction, but both the rate and the total amount of resorption may vary between individuals. While the causes of this variation are still unclear, it is recognised that resorption of residual ridges after loss of all the teeth is a complex biophysical process. Successful replacement of the dentition with complete removable dentures that merely rest on the mucosa presents a challenge, not only for dentists but for the wearer: in order to eat, drink, or talk whilst wearing dentures, patients must master amazing adaptations of the oral musculature (Fig. 32.1).

The concept of treating edentulism by osseointegration of dental implants was first proposed in the 1960s by two independent groups: Professor Schroeder at the University of Berne, Switzerland and Professor Brånemark at the University of Gothenburg, Sweden. Their data were based on treatment protocols using endosseous, root analogue, titanium implants. These investigators were the first to document the fundamental requirements for osseointegration and the interaction between the titanium surface and bone (Brånemark et al. 1969, 1977; Schroeder et al. 1981). They also addressed the primary biomechanical requirements for dental implant design. Both research teams obtained excellent results through the integration of basic biological and biomechanical knowledge and the initiation and application of clinical research projects.

Most of the endosseous cylindrical implant systems subsequently developed, both for submerged and non-submerged implant procedures, followed the guidelines for successful osseointegration by Adell et al. (1981), *i.e.* a 3–6-month unloaded healing period. It was argued that implants required an undisturbed healing time for successful tissue integration and that premature loading might prevent

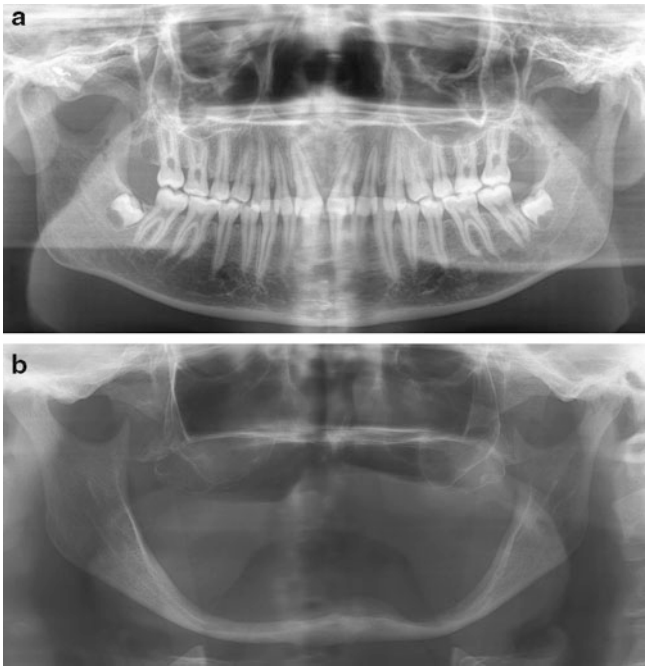


Fig. 32.1 Radiograph of fully dentate jaws with no signs of bone loss or other defects (**a**). Advanced resorption of the mandible in an edentulous patient (**b**)

direct bone apposition and lead to fibrous tissue encapsulation. Improved understanding of the osseointegration process, bone resorption and re-modelling and the interaction between bone and metal surfaces has resulted in recent departures from the traditional conservative approach established some 40 years ago. The importance of the surface characteristics and choice of the implant material in determining the quality of bone anchorage was recognized early (Albrektsson et al. 1981; Buser et al. 1991; Johansson 1991). Various surface treatments have been successfully used to achieve more rapid and more stable bone integration *i.e.* bone-metal anchorage (Buser et al. 1999; Albrektsson et al. 2000; Arvidson 1998; Arvidson et al. 1998, 2008; Fischer et al. 2008, for recent reviews see, Esposito et al. 2004, 2007a, b; Wennerberg and Albrektsson 2009).

Successful endosseous implantation in the alveolar ridge requires sufficient quality and quantity of bone at the recipient site. Several surgical techniques have been described to augment bone before or in combination with dental implant installation (for a review see Hammerle and Jung 2003). More recently, in a relatively limited RCT study, Jung et al. (2009) demonstrated that implants installed in defective bone sites grafted with demineralised bovine mineral with or without a growth factor (rhBMP-2) had excellent clinical and radiological outcomes after 5 years.

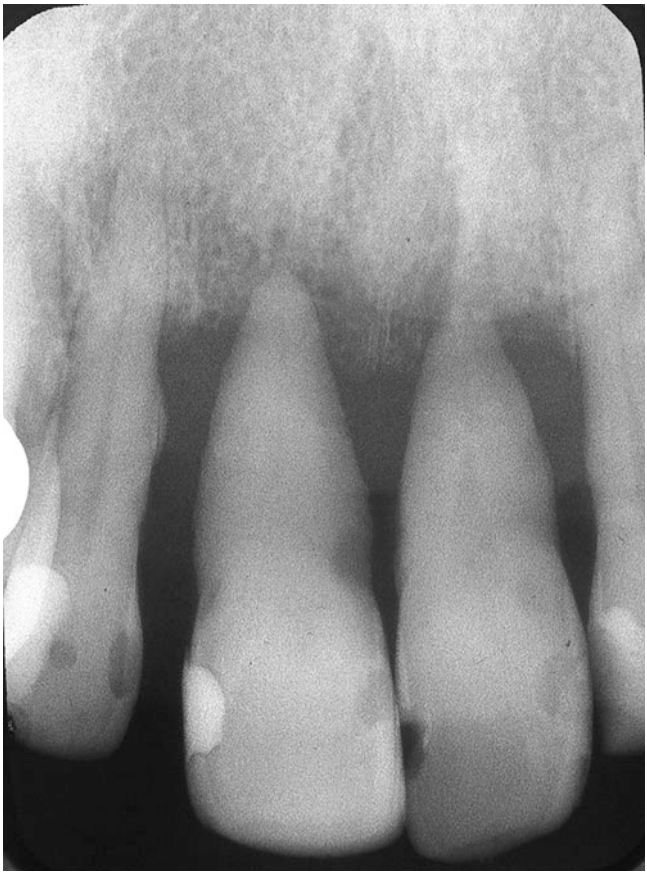


Fig. 32.2 Radiograph of very severe bone loss around the maxillary anterior teeth

32.2.2 Loss of Periodontal Tissues

The main function of the periodontium is to attach the tooth to the alveolar bone and to maintain the surface integrity of the masticatory mucosa. Epidemiological studies have shown that infections are the main cause of destruction of bone as a supporting tissue of the teeth. The etiology has, however, been shown to be multicausal. Periodontal disease, especially the most severe forms, is no longer regarded as a simple infection, but rather as the result of a complicated interaction with systemic factors or disorders. In the most severe cases the outcome can be the loss of most or all teeth (Fig. 32.2).

An important goal of periodontal therapy is to achieve a reduction in the depth of the periodontal pocket in order to prevent further disease progression. In patients with moderate periodontitis, *i.e.* pocket depths ≤6 mm, this goal can be accomplished

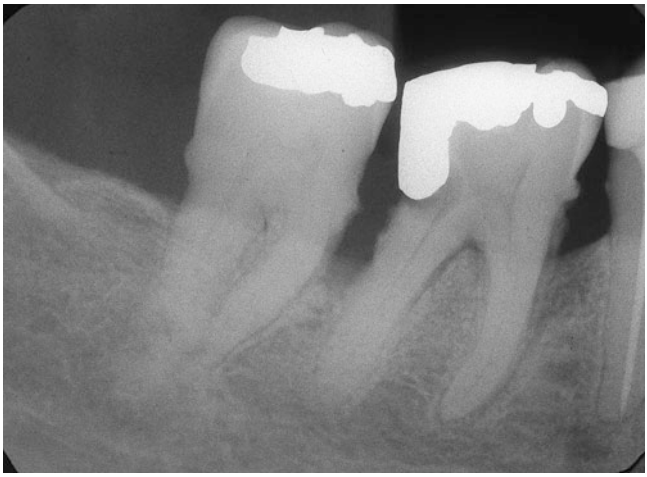


Fig. 32.3 Radiograph of vertical bone loss around a mandibular molar

by non-surgical therapy, whereas in severe cases, particularly in the presence of intrabony defects and furcations (Fig. 32.3), the treatment must be supplemented with periodontal surgery. There is increasing use of regenerative procedures to restore lost periodontal support.

Periodontal regeneration has been defined as the process by which the architecture and function of the periodontal tissues are completely renewed (The American Academy of Periodontology 1992) and includes the formation of a new connective tissue attachment, cementum and supporting bone (Ellegaard et al. 1973, 1974; Karring et al. 1993). Regenerative periodontal therapy comprises procedures which are specially designed to restore, by reattachment or new attachment, those parts of the supporting apparatus which have been lost due to periodontitis, *i.e*. gingiva, periodontal ligament, root cementum and alveolar bone. For true regeneration, the root surface must therefore be repopulated by epithelial cells and cells derived from the gingival connective tissue, bone and periodontal ligament. Guided tissue regeneration (GTR) is a treatment modality intended to promote regeneration of periodontal tissue lost through periodontitis. Animal studies have confirmed that in intra-bony defects, this treatment results in true regeneration, albeit with some limitations (Laurell et al. 2006). GTR has also been used in implant rehabilitation, using different techniques and membrane materials (for a review see Hammerle and Jung 2003).

The most commonly used clinical methods for regeneration of the periodontal attachment apparatus are GTR (Sculean et al. 2008) and a derivative of enamel matrix proteins (EMD). GTR, using bioabsorbable barriers made of e.g. polylactide acetyltributyl citrate or polydioxanon, has shown stable clinical results in both short and long term studies (Eickholz et al. 2004). EMD are acidic extracts of extracellular enamel matrix, and include a heterogeneous mixture of polypeptides encoded

by several genes (Bosshardt 2008). It is unclear which of the enamel matrix proteins induces the regeneration, and the underlying molecular mechanisms have yet to be determined.

The use of bioactive molecules to induce local bone formation is an active field of research. Bioactive agents are used alone or together with grafting or GTR for treatment of intra-osseous and furcation defects (Trombelli and Farina 2008). A variety of growth factors have also been tested for local bone regeneration (for a recent systematic review see Jung et al. 2008).

32.2.3 Loss of Bone

Bone defects in the oral and maxillo-facial region may arise following surgical treatment of tumors, cysts and other pathological conditions as well as traumatic insults to the facial and dento-alveolar structures. As such defects often involve structures of different origins, the reconstructive procedures are very demanding. Maxillofacial tumors and cysts may arise from both soft and hard tissues and may be of odontogenic or nonodontogenic origin. Lesions located within the jaws thus include odontogenic cysts and tumors, nonodontogenic cysts and benign tumors and malignant, nonodontogenic neoplasms. Benign cysts and tumors occur frequently, are clinically and radiologically well-delineated and treated by curettage or enucleation, whereas highly proliferative lesions are treated by resection. Malignant primary neoplasms of the jaws are rare, the most common being osteosarcoma. Much less common are chondrosarcoma, plasmocytoma and Ewing's sarcoma. Some of these tumors may require extensive surgical treatment and reconstruction. Further examples of pathological conditions of the jaws requiring treatment by extensive bone resection are osteoradionecrosis or extensive, proliferative benign lesions which have proved resistant to other therapies. The most frequent pathological conditions for extensive resection and reconstruction however, are malignant tumors in the oral cavity and maxillary sinus, invading bone tissue.

Despite progress in the field of reconstruction as a result of new surgical techniques, improved biomaterials and advances in cell biology, autologous bone grafting remains the "gold standard", especially for the reconstruction of large bone defects (Chiapasco et al. 2008; Raveh et al. 1987) (Figs. 32.4 and 32.5).

Free, nonvascularized autologous transplants are functioning for bridging of defects and as volume fillers by inducing bone growth. In some cases, however, the prognosis may be guarded, due to the risk of inadequate vascular regeneration and impaired tissue repair following hypoxia. Of vital importance to success are adequate microvascularity of the recipient tissues and optimal fixation of the grafts, in order to prevent infection and loss of osteogenic cells. Segmental osteodistraction may have potential as a treatment solution. In cases of compromised tissue healing or composite tissue defects, the treatment of choice is the use of revascularized hard and soft tissue free flaps (Torroni et al. 2007; Smolka and Iizuka 2005; Emerick and Teknos 2007; Chepeha et al. 2008; Chiapasco et al. 2006). Despite the above risk

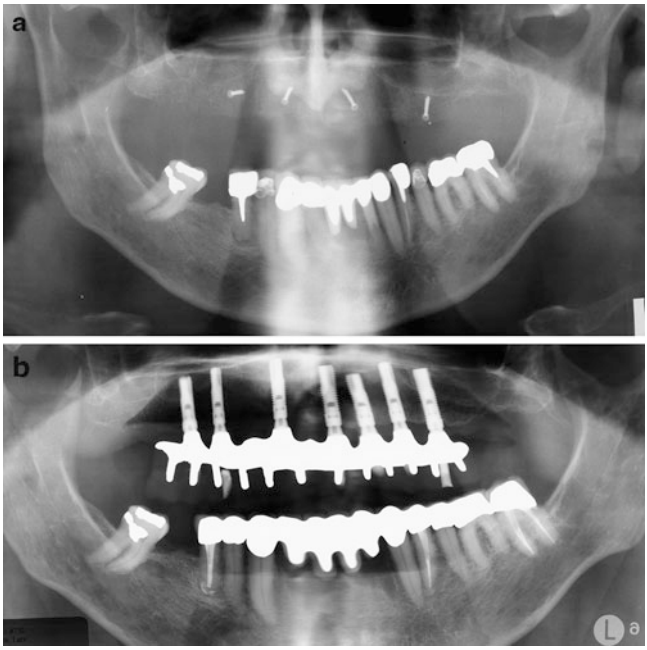


Fig. 32.4 Edentulous maxilla with extensive resorption of the alveolar crest. Reconstructed with free cortical onlay blocks from the iliac crest, fixed with miniscrews (**a**). After a healing period, a full arch maxillary bridge was retained on seven osseointegrated implants (**b**)

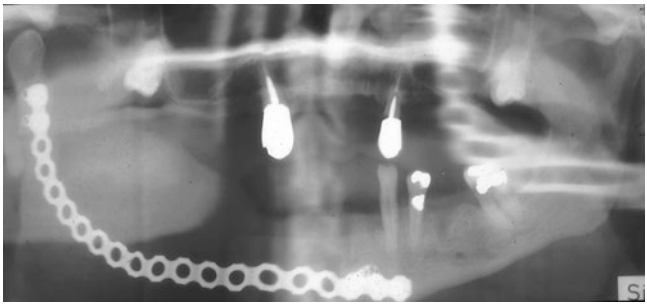


Fig. 32.5 Mandibular defect after tumor resection. Treated with a free revascularized forearm soft tissue graft and a bridging reconstruction plate

factors, good functional and esthetic outcomes have been reported (Chiapasco et al. 2008; Louis et al. 2008) (Figs. 32.6 and 32.7).

For reconstruction of minor and single tissue defects, a wide range of autografts, allografts, xenografts and synthetic substitutes has been extensively used in recent years, in some instances showing outcomes comparable with autologous grafts (Hallman and Thor 2008; Hellem et al. 2003). In a review article Kretlow et al. (2009)

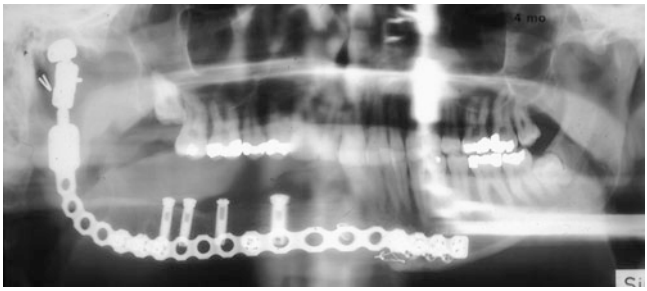


Fig. 32.6 Hemi-mandibular defect after tumor resection. Treated with a temporo-mandibular joint prosthesis, a bridging reconstruction plate with free iliac crest graft and osseointegrated dental implants

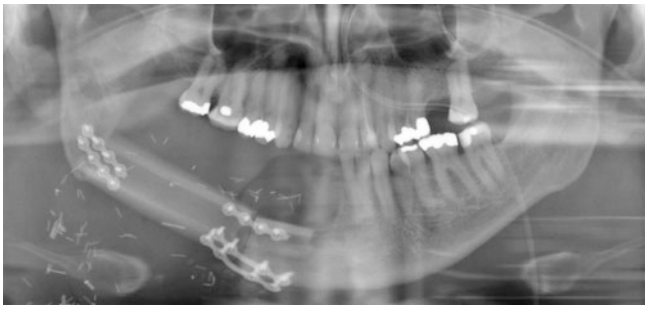


Fig. 32.7 Lateral mandibular defect after tumor resection. Treated with a free revascularized compound fibular graft

presented an excellent summary of newer materials and methods in bone and soft tissue regeneration. Compared with autologous transplants, the disadvantages of allografts, xenografts and synthetic biomaterials include lack of osteoinductive properties and relatively varying osteoconduction. Varying, and to some degree uncontrolled resorption rates may represent a challenge in a clinical situation when assessing the amount and progression of tissue regeneration. The risks of bacterial, viral or prion transmission from allo- and xenografts as well as immunologic reactions are minimal and dependent on the method used for tissue preservation (Kretlow et al. 2009).

Bone and soft tissue defects due to high velocity insults may be extensive, and involve several areas of tissue loss and progressive necrosis, demanding extensive surgery. These defects often have to be reconstructed by two stage surgery, using revascularized free or pedicled compound flaps or osteodistraction (Bertelè et al. 2005; Pereira et al. 2007). Defects due to non-optimal repositioning of fractures in the periorbital, naso-ethmoidal and midface regions still remain a challenge, the mandibular regions however not.

Severe dento-alveolar trauma occurring in isolation or in combination with facial trauma, is often associated with loss of teeth and bone defects in the alveolar crest. Cases involving primary or secondary loss of teeth and bone tissue have to be reconstructed as a prerequisite for treatment with dental implants. In some cases replacement of lost mucosal/gingival soft tissue must be addressed as well. Functional and esthetic outcomes are priorities for treatment of bone defects in the maxillary anterior alveolar crest. Bone grafting and local osteodistraction (Lundgren and Sennerby 2008) or even non resorbable bone substitutes are treatment modalities (Hallman et al. 2009; Hellem et al. 2003).

32.3 Future Directions

32.3.1 Oral Stem Cells in Regenerative Dentistry

Physiological bone tissue regeneration is a remarkable process that results in healing without scarring. It is a multi-faceted process, beginning with angiogenesis, followed by callus formation and eventually bone remodelling. Key contributing factors in this process are growth factors [VEGF, PDGF-BB, plGF, BMPs, basic Fibroblast Growth Factor (bFGF)] osteocytes and angiocytes of the surrounding bone tissue, adult mesenchymal and hematopoietic stem cells. However, the prognosis is uncertain in the presence of large defects (>1 cm) or conditions associated with healing impairment such as old age, diabetes or radiation therapy. Under such suboptimal conditions, the gold standard of autologous bone transplantation is however associated with disadvantages, such as the limited amount of bone which can be harvested, unpredictable donor bone turnover, donor site morbidity, and the added cost incurred by surgical procedures to harvest the bone as well as pain at the harvest site.

Currently, various strategies are applied to stimulate healing of bone defects and to restore lost maxillofacial bone and periodontal support following traumatic insult, tumor ablation, diseases or congenital deformities. Despite the fact that materials science and technology has markedly improved the field of bone regeneration, none of the currently available treatment regimes stimulates bone and attachment formation. They therefore lack the potential to increase bone density and volume significantly and to form a new, functional periodontal attachment. For this reason large defects/injuries still represent a major challenge for dentists and oral maxillofacial surgeons. The clinical challenges have stimulated interest in developing new therapies that involve regeneration of bone and periodontal ligament.

Bone marrow has been shown to contain a population of rare cells capable of differentiating into the cells that form various tissues. These cells, referred to as mesenchymal stem cells (MSC), are located within the bone marrow and, depending on the culture conditions chosen, have the potential to differentiate into fibroblastic, osteogenic, adipogenic or reticular cells (Friedenstein 1976; Bianco et al. 2001). The lack of immunogenicity of MSC heightens the potential of these cells for bone repair. Human bone marrow osteoprogenitors can be isolated and enriched from the CD34+

fraction using selective markers such as STRO-1 (Stewart et al. 1999). In recent years there has been increasing interest in the possibility of using adult MSC for regeneration of oral tissues, not only to enhance attachment around periodontally compromised teeth, but also to augment alveolar bone before and/or after placement of oral implants. Adult stem cells, previously thought to be limited in potential, have increasingly been shown to be able to differentiate into tissues of an entirely different germ layer, with potential clinical application in the treatment of a number of diseases.

One of the most extensively studied populations of pluripotent stem cells has been mesenchymal stem cells (MSC) from bone marrow. It has been shown that from a small volume (0.1–3 ml) of marrow aspirate, alveolar bone mesenchymal cells (BMSC) can be expanded successfully 70% of the time (Matsubara et al. 2005). Alveolar BMSC might be useful for regenerative medicine, because small marrow aspirates from alveolar bone can be made with minimal pain. Furthermore, Matsubara et al. (2005) demonstrated a high osteogenic potential from alveolar BMSC. Although this raises few ethical issues, harvesting of cells from bone marrow is still an invasive procedure, and stem cell numbers decrease significantly with the age. The search for more readily accessible sources of pluripotent stem cells has led to investigation of other tissues, including mobilized peripheral blood, umbilical cord blood and more recently, periodontal ligament (PDL), deciduous and permanent teeth.

The PDL is one of the tissues that has attracted interest as a source of stem cells and its potential for regeneration. It contains a heterogeneous cell population that can differentiate into cementoblasts or osteoblasts. Recent findings suggest that PDL cells have osteoblast-like properties. They have the capacity to form mineralized nodules *in vitro*, express bone-associated markers such as alkaline phosphatase and sialoprotein, and also respond to bone inductive factors such as parathyroid hormone, insulin -like growth factor 1, bone morphogenetic protein 2, and transforming growth factor □$_1$. Seo et al. (2004) showed that human PDL cells participate in periodontal tissue repair in immunocompromised rats, indicating that the PDL contains stem cells.

Dental pulp tissue is also a readily accessible source of pulp-derived mesenchymal stem cells (PDSC). PDSC express the endothelial and smooth muscle marker STRO-1 (Shi and Gronthos 2003) and display a pericyte phenotype, with expression of the pericyte-associated antigen 3G5 (Shi and Gronthos 2003). It is therefore assumed, but not yet confirmed, that the perivascular region in the pulp is the niche for PDSC and that pericytes give rise to dental pulp stem cells. Isolated dental pulp stem cells have been shown to be plastic-adherent and express the MSC markers STRO-1, CD90, CD29, CD44, CD166, CD105, CD106, CD146, CD13 and are also negative for CD14 and CD34 (Shi et al. 2005; Ikeda et al. 2006). *In vitro*, PDSC are capable of self-renewal, display plasticity and mutilineage potential (adipocytes, chondrocytes, osteoblasts, neural cell progenitors and myotubes) and can therefore be considered as stem cells (Gronthos et al. 2002).

For tissue engineering purposes, PDSC have shown potential for both dentin and bone production. From the pool of human dental pulp cells, odontoblasts capable of forming dentin-like structures can be differentiated when cultured under mineralization-enhancing conditions (About et al. 2000). Moreover, in immunocompromised mice, subcutaneously implanted cells derived from human dental pulp generate a dentin-pulp-like

complex without lamellar bone (Shi et al. 2005). Using a similar model, another research group has also shown that PDSC are able to generate vascularized bone tissue that *in vivo* was remodelled into a lamellar bone (Laino et al. 2005, 2006a, b; d'Aquino et al. 2007). Further, when implanted into immunocompromised rats, a distinguishable STRO-1 positive subpopulation of cells was found to produce woven bone efficiently and to remodel lamellar tissue (d'Aquino et al. 2007; Laino et al. 2006b). After implantation, PDSC expressed bone markers including osteocalcin, Runx-2, collagen I and alkaline phosphatase (d'Aquino et al. 2007). Furthermore, it might be possible for PDSC to contribute to the formation of new bone containing Haversian channels with appropriate vascularization *in vivo* (Huang et al. 2008; Pierdomenico et al. 2005; Shi et al. 2005; Young et al. 2002; d'Aquino et al. 2007; Laino et al. 2006b; Ikeda et al. 2006; Gronthos et al. 2000; About et al. 2000; Batouli et al. 2003; Cheng et al. 2008). Even when removed from their native location, dental pulp cells maintain the potential to contribute to the formation of both dentin and alveolar bone (Diep et al. 2009).

The transition from deciduous (baby) teeth to permanent (adult) teeth is a unique, dynamic process in which the development and eruption of the permanent teeth is co-ordinated with the resorption of the roots of deciduous teeth. In humans, it may take >7 years to complete the orderly replacement of 20 deciduous teeth. Recently, researchers found that a naturally exfoliated human deciduous tooth contains a population of stem cells (SHED) and are thus available without surgical intervention (Laino et al. 2006b). These cells have been shown to be plastic-adherent, have great proliferative capacity and positive for MSCs markers STRO-1, CD29, CD106, CD146, while negative for CD14, CD34 (Shi et al. 2005). Further, they exhibited a high degree of plasticity with the capacity to differentiate into neurons, adipocytes, osteoblasts and odontoblasts (Miura et al. 2003; Huang et al. 2008). SHED are not only derived from a very accessible tissue resource but are also capable of providing enough cells for potential clinical application. Thus, exfoliated teeth may be an unexpected, unique resource for stem cell therapies including autologous stem cell transplantation and tissue engineering. These cells could aid the repair of damaged teeth and perhaps even treat neural injuries or degenerative diseases. Stem cells isolated from deciduous teeth (SHED) have several advantages. Although unlikely to have the differentiation and proliferative potential of ESC, deciduous tooth stem cells require no invasive harvesting procedure. Furthermore, there are no ethical issues, as in the normal course of events deciduous teeth exfoliate and are discarded.

32.3.2 Artificial Scaffolds in Regenerative Dentistry

The concept of tissue engineering has emerged as a valid approach to current therapies for bone regeneration. In contrast to the conventional biomaterials approach, tissue engineering is based on an understanding of tissue formation and regeneration, and aims at inducing new functional tissues, rather than just implanting replacement parts. There are numerous published case reports of the application of bone tissue

engineering for oral and maxillofacial surgical reconstruction, periodontal tissue regeneration and sinus floor augmentation. Tissue engineering is the application of scientific principles to the design, construction, modification and growth of living tissues, using biomaterials, cells, and factors alone or in combination. Skeletal tissue engineering requires a scaffold conducive to cell attachment and maintenance of cell function, in combination with a rich source of osteoprogenitor cells and osteoinductive growth factors. Crucial to success is an understanding of how cells function and form a matrix, and the development of appropriate materials for fabrication of scaffolding designed to promote cell attachment and maintain cell function.

Recently, much effort has been devoted to synthesis methods and fabrication techniques used to design and select a scaffold with properties that most closely match those required for bone regeneration. Highly porous and degradable aliphatic polyester scaffolds with varying pore size and interconnected pores were fabricated by bulk copolymerization of poly(L-lactide) (PLLA), 1,5-dioxepan-2-one (DXO-co-LLA) and ϵ-caprolactone (CL-co-LLA) (Dånmark et al. 2010). The degradation rates of polyester scaffolds and loss of mechanical integrity were greatly increased in porous scaffolds made with hydrophilic co-monomers (Dånmark et al. 2011). By incorporating hydrophobic co-monomers with limited ability to crystalize instead of hydrophilic co-monomers, the mechanical stability was retained longer during degradation. It has been shown that these scaffolds are biocompatible and stimulate bone regeneration both *in vitro* and *in vivo* (Arvidson et al. 2011; Dånmark 2011; Idris 2010; Xing 2012; Xue 2011). These polyester scaffolding materials show great potential as bone tissue constructs. However, the scaffolds need to be optimized to control cell differentiation and growth as well as to achieve angiogenesis before they are ready for human use.

32.3.3 Paracrine Effects of Stem Cell-Derived Growth Factors

Tooth regeneration by cell transplantation is a meritorious approach. However, there are hurdles in the translation of cell-delivery-based tooth regeneration into therapeutics. The inaccessibility of autologous embryonic tooth germ cells for human applications, the limited availability of autologous postnatal tooth germ cells (e.g. third molars) and the low survival rates of the implanted cells may undermine the efficacy of the cell-based treatment. Furthermore, other factors such as the availability of autologous dental pulp stem cells, the excessive costs of cell isolation, handling, storage, shipping and *ex vivo* manipulation, liability issues if contamination occurs, and potential for transmission of infectious disease are all potential drawbacks to cell transplantation (Inanc and Elcin 2011; Yildirim et al. 2011).

It has been reported that stem cells secrete multiple metabolites, growth factors, signaling molecules, and extracellular matrix proteins during *in vitro* culture that affect cellular behavior (Kinnaird et al. 2004; Barcelos et al. 2009; Cai et al. 2009; Perin and Silva 2009; Osugi et al. 2012). Stem cell-conditioned medium (CM) can be used, transplanted or injected with or without scaffolds to induce cell homing, migration, proliferation and differentiation (Ueda and Nishino 2010; Kim et al.

2009; Yang et al. 2009). Therefore, the use of stem cell-conditioned medium as an alternative to transplanting stem cells might be a feasible approach for tissue engineering. The paracrine effects of the growth factors in CM on recruiting circulating progenitor/stem cells and/or endogenous adjacent cells to the treatment site is attracting considerable research attention at present. Although the molecular mechanisms that direct mobilization and homing of cells in response to the paracrine factors secreted by stem cells are not fully understood, cell homing represents a novel concept for regenerative dentistry and may offer a clinically useful approach (Kim et al. 2010).

The therapeutic effects of CM derived from stem cells derived from different sources have been demonstrated in experimental animal models (Cho et al. 2012; Osugi et al. 2012). It has been shown that conditioned medium derived from mesenchymal stem cells as well as SHED-conditioned medium is able to accelerate wound healing as well as tat seen with stem cell transplantation, and thus may become a new therapeutic method for wound healing in the future (Tamari et al. 2011; Ueda and Nishino 2010). Thus conditioned medium might be used to create a highly inductive microenvironment, with many possible uses in regenerative dentistry. However, further studies are required to address the underlying mechanisms involved in organogenesis mediated by conditioned medium.

32.4 Conclusion

PDL, PDSC, SHED and alveolar bone mesenchymal stem cells appear to be appropriate candidates for tissue engineering involving restoration of dental and periodontal tissues, as well as bone, suggesting a potential future therapeutic role of these cells for craniofacial regeneration. Artificial scaffolds are currently underdevelopment and may, together with cells from these different sources, lead to improvements in tissue engineering of bone defects in the oral cavity. The use of paracrine factors to improve tissue regeneration is a very promising new concept. However, much work remains before this approach will be ready for routine clinical use.

References

About I, Bottero MJ, de Denato DP, Camps J, Franquin JC, Mitsiadis TA (2000) Human dentin production in vitro. Exp Cell Res 10:33–41

Adell R, Lekholm U, Rockler B, Brånemark P-I (1981) A 15-year study of osseointegrated implants in the treatment of the edentulous jaw. Int J Oral Surg 10:387–416

Albrektsson T, Brånemark P-I, Hansson HA, Lindström J (1981) Osseointegrated titanium implants. Requirements for ensuring a long-lasting, direct bone-to-implant anchorage in man. Acta Orthop Scand 52:155–170

Albrektsson T, Johansson C, Lundgren AK, Sul YT, Gottlow J (2000) Experimental studies on oxidized implants. A histomorphometrical and biomechanical analysis. Appl Osseointegr Res 1:21–24

Arvidson K (1998) A subsequent two-stage dental implant system and its clinical application. Periodontol 2000 2000(17):96–105

Arvidson K, Bystedt H, Frykholm A, von Konow L, Lothigius E (1998) Five-year prospective follow-up report of the Astra Tech Dental Implant System in the treatment of edentulous mandibles. Clin Oral Implants Res 9:225–234

Arvidson K, Esselin O, Felle-Persson E, Jonsson G, Smedberg JI, Soderstrom U (2008) Early loading of mandibular full-arch bridges screw retained after 1 week to four to five Monotype implants: 3-year results from a prospective multicentre study. Clin Oral Implants Res 19:693–703

Arvidson K, Abdallah BM, Applegate LA, Baldini N, Cenni E, Gomez-Barrena E, Granchi D, Kassem M, Konttinen YT, Mustafa K, Pioletti DP, Sillat T, Finne-Wistrand A (2011) Bone regeneration and stem cells. Regenerative medicine review series. J Cell Mol Med 15:718–746

Barcelos LS, Duplaa C, Kränkel N, Graiani G, Invernici G, Katare R, Siragusa M, Meloni M, Campesi I, Monica M, Simm A, Campagnolo P, Mangialardi G, Stevanato L, Alessandri G, Emanueli C, Madeddu P (2009) Human CD133+ progenitor cells promote the healing of diabetic ischemic ulcers by paracrine stimulation of angiogenesis and activation of Wnt signaling. Circ Res 104:1095–1102

Batouli S, Miura M, Brahim J, Tsutsui TW, Fisher LW, Gronthos S, Robey PG, Shi S (2003) Comparison of stem-cell-mediated osteogenesis and dentinogenesis. J Dent Res 82:976–981

Bertelè G, Mercanti M, Stella F, Albanese M, De Santis D (2005) Osteodistraction in the craniofacial region. Minerva Stomatio 54:179–198

Bianco P, Riminucci M, Gronthos S, Robey PG (2001) Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 19:180–192

Bosshardt DD (2008) Biological mediators and periodontal regeneration: a review of enamel matrix proteins at the cellular and molecular levels. J Clin Periodontol 35:87–105

Brånemark P-I, Adell R, Breine U, Hansson BO, Lindström J, Ohlsson A (1969) Intra-osseeous anchorage of dental prostheses. I. Experimental studies. Scand J Plast Reconstr Surg Hand Surg 3:81–100

Brånemark P-I, Hansson BO, Adell R, Breine U, Lindström J, Hallen O, Öhman A (1977) Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year period. Scand J Plast Reconstr Surg Suppl 16:1–132

Buhlin K, Gustafsson A, Hakansson J, Klinge B (2002) Oral health and cardiovascular disease in Sweden. J Clin Periodontol 29:254–259

Buhlin K, Gustafsson A, Pockley AG, Frostegard J, Klinge B (2003) Risk factors for cardiovascular disease in patients with periodontitis. Eur Heart J 24:2099–2107

Buser D, Schenk RK, Steinemann S, Fiorellini J, Fox C, Stich H (1991) Influence of surface characteristics on bone integration of titanium implants. A histomorphometric study in miniature pigs. J Biomed Mater Res 25:889–902

Buser D, Nydegger T, Oxland T, Cochran DL, Schenk RK, Hirt HP, Snetivy D, Nolte LP (1999) Interface shear strength of titanium implants with a sandblasted and acid-etched surface: a biomechanical study in the maxilla of miniature pigs. J Biomed Mater Res 45:75–83

Cai L, Johnstone BH, Cook TG, Tan J, Fishbein MC, Chen PS, March KL (2009) IFATS collection: human adipose tissue-derived stem cells induce angiogenesis and nerve sprouting following myocardial infarction, in conjunction with potent preservation of cardiac function. Stem Cells 27:230–237

Cheng PH, Snyder B, Fillos D, Ibegbu CC, Huang AH, Chan AW (2008) Postnatal stem/progenitor cells derived from the dental pulp of adult chimpanzee. BMC Cell Biol 9:20

Chepeha DB, Teknos TN, Fung K, Shargordosky J, Sacco AG, Nussenbaum B, Jones L, Eisbruch A, Bradford CR, Prince ME, Moyer JS, Lee LS, Wolf GT (2008) Lateral oromandibular defect: when is it appropriate to use a bridging reconstruction plate combined with a soft tissue revascularized flap? Head Neck 30:709–717

Chiapasco M, Biglioli F, Autelitano L, Romeo E, Bursati R (2006) Clinical outcome of dental implants placed in fibula-free flaps used for the reconstruction of maxillo-mandibular defects following ablation for tumors and osteoradionecrosis. Clin Oral Implants Res 17:220–228

Chiapasco M, Colletti G, Romeo E, Zaniboni M, Brusati R (2008) Long-term results of mandibular reconstruction with autogenous bone grafts and oral implants after tumor resection. Clin Oral Implants Res 19:1074–1080

Cho YJ, Song HS, Bhang S, Lee S, Kang BG, Lee JC, An J, Cha CI, Nam DH, Kim BS, Joo KM (2012) Therapeutic effects of human adipose stem cell-conditioned medium on stroke. J Neurosci Res 90(9):1794–1802, Apr 26

d'Aquino R, Graziano A, Sampaolesi M, Laino G, Pirozzi G, De Rosa A, Papaccio G (2007) Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation. Cell Death Differ 14:1162–1171

Dånmark S (2011) Designing biodegradable polymer scaffolds aimed for tissue engineering. Ph.D. thesis. Department of Clinical Dentistry, University of Bergen, Norway

Dånmark S, Wistrand A, Albertsson A-C, Wendel M, Arvidson K, Mustafa K (2010) Osteogenic differentiation in rat bone marrow derived stromal cells on refined biodegradable polymer scaffolds. J Bioact Compat Polym 25:207–223

Dånmark S, Finne-Wistrand A, Schander K, Hakkarainen M, Arvidson K, Mustafa K, Albertsson AC (2011) In vitro and in vivo degradation profile of aliphatic polyesters subjected to electron beam sterilization. Acta Biomater 7:2035–2046

Diep L, Matalova E, Mitsiadis TA, Tucker AS (2009) Contribution of the tooth bud mesenchyme to alveolar bone. J Exp Zoolog B Mol Dev Evol 15:510–517

Eickholz P, Krigar DM, Pretzl B, Steinbrenner H, Dorfer C, Kim TS (2004) Guided tissue regeneration with bioabsorbable barriers. II. Long-term results in infrabony defects. J Periodontol 75:957–965

Ellegaard B, Karring T, Listgarten M, Löe H (1973) New attachment after treatment of interradicular lesions. J Periodontol 44:209–217

Ellegaard B, Karring T, Davies R, Löe H (1974) New attachment after treatment of intrabony defects in monkeys. J Periodontol 45:368–377

Emerick KS, Teknos TN (2007) State-of-the-art mandible reconstruction using revascularized free-tissue transfer. Expert Rev Anticancer Ther 7:1781–1788

Esposito M, Worthington HV, Thomsen P, Coulthard P (2004) Interventions for replacing missing teeth: different times for loading dental implants. Cochrane Database Syst Rev (3). Art. No.: CD003878.pub2. DOI: 10.1002/14651858.CD003878.pub2

Esposito M, Murray-Curtis L, Grusovin MG, Coulthard P, Worthington HV (2007a) Interventions for replacing missing teeth: different types of dental implants. Cochrane Database Syst Rev (4). Art. No.: CD003815. DOI: 10.1002/14651858.CD003815.pub3

Esposito M, Grusovin MG, Maghaireh H, Coulthard P, Worthington HV (2007b) Interventions for replacing missing teeth: management of soft tissues for dental implants. Cochrane Database Syst Rev (3). Art. No.: CD006697. DOI: 10.1002/14651858.CD006697

Fischer K, Stenberg T, Hedin M, Sennerby L (2008) Five-year results from a randomized, controlled trial on early and delayed loading of implants supporting full-arch prosthesis in the edentulous maxilla. Clin Oral Implants Res 19:433–441

Friedenstein AJ (1976) Precursor cells of mechanocytes. Int Rev Cytol 47:327–359

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA 5:13625–13630

Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, Denbesten P, Robey PG, Shi S (2002) Stem cell properties of human dental pulp stem cells. J Dent Res 81:531–535

Hallman M, Thor A (2008) Bone substitutes and growth factors as an alternative/complement to autogenous bone for grafting in implant dentistry. Periodontol 2000 2000(47):172–192

Hallman M, Mordenfeld A, Strandkvist T (2009) Bone replacement following dental trauma prior to implant surgery – present status. Review article. Dent Traumatol 25:2–11

Hammerle CHF, Jung RE (2003) Bone augmentation by means of barrier membranes. Periodontol 2000 2000(33):36–53

Hellem S, Åstrand P, Stenstrom B, Engquist B, Bengtsson M, Dahlgren S (2003) Implant treatment in combination with lateral augmentation of the alveolar process: a 3-year prospective study. Clin Implant Dent Relat Res 5:233–240

Huang GT, Sonoyama W, Liu Y, Liu H, Wang S, Shi S (2008) The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering. J Endod 34:645–651

Idris S (2010) Biological responses to aliphatic polyester scaffolds for bone tissue engineering applications. Ph.D. thesis. Department of Clinical Dentistry, University of Bergen, Norway

Ikeda E, Hirose M, Kotobuki N, Shimaoka H, Tadokoro M, Maeda M, Hayashi Y, Kirita T, Ohgushi H (2006) Osteogenic differentiation of human dental papilla mesenchymal cells. Biochem Biophys Res Commun 342:1257–1262

Inanc B, Elcin YM (2011) Stem cells in tooth tissue regeneration-challenges and limitations. Stem Cell Rev 7:683–692

Johansson C (1991) On tissue reactions to metal implants. Ph.D. thesis. Biomaterials Group, Department of Handicap Research, University of Gothenburg, Sweden

Jung RE, Thoma DS, Hammerle CHP (2008) Assessment of the potential of growth factors for localized alveolar ridge augmentation: a systematic review. J Clin Periodontol 35:255–281

Jung RE, Windisch SI, Eggenschwiler AM, Thoma DS, Weber FE, Hammerle CHF (2009) A randomized-controlled clinical trial evaluating clinical and radiological outcomes after 3 and 5 years of dental implants placed in bone regenerated by means of GBR techniques with or without the addition of BMP-2. Clin Oral Implants Res 20:660–666

Karring T, Nyman S, Gottlow J, Laurell L (1993) Development of the biological concept of guided tissue regeneration-animal and human studies. Periodontol 2000 2000(1):26–35

Kim HS, Lee DS, Lee JH, Kang MS, Lee NR, Kim HJ, Ko JS, Cho MI, Park JC (2009) The effect of odontoblast conditioned media and dentin non-collagenous proteins on the differentiation and mineralization of cementoblasts in vitro. Arch Oral Biol 54:71–79

Kim JY, Xin X, Moioli EK, Chung J, Lee CH, Chen M, Fu SY, Koch PD, Mao JJ (2010) Regeneration of dental-pulp-like tissue by chemotaxis-induced cell homing. Tissue Eng Part A 16:3023–3031

Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, Epstein SE (2004) Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote *in vitro* and *in vivo* arteriogenesis through paracrine mechanisms. Circ Res 94:678–685

Kretlow JD, Young S, Klouda L, Wong M, Mikos AG (2009) Injectable biomaterials for regenerating complex craniofacial tissues. Adv Mater 21:3368–3393

Laino G, d'Aquino R, Graziano A, Lanza V, Carinci F, Naro F, Pirozzi G, Papaccio G (2005) A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). J Bone Miner Res 20:1394–1402

Laino G, Carinci F, Graziano A, d'Aquino R, Lanza V, De Rosa A, Gombos F, Caruso F, Guida L, Rullo R, Menditti D, Papaccio G (2006a) *In vitro* bone production using stem cells derived from human dental pulp. J Craniofac Surg 17:511–515

Laino G, Graziano A, d'Aquino R, Pirozzi G, Lanza V, Valiante S, De Rosa RA, Naro F, Vivarelli E, Papaccio G (2006b) An approachable human adult stem cell source for hardtissue engineering. J Cell Physiol 206:693–701

Laurell L, Bose M, Graziani F, Tonetti M, Berglundh T (2006) The structure of periodontal tissue formed following guided tissue regeneration therapy of intra-bony defects in the monkey. J Clin Periodontal 33:596–603

Louis PJ, Gutta R, Said-Al-Naief N, Bartolucci AA (2008) Reconstruction of the maxilla and mandible with particulate bone graft and titanium mesh for implant placement. J Oral Maxillofac Surg 66:235–245

Lundgren S, Sennerby L (2008) Bone reformation. Contemporary bone augmentation procedures in oral and maxillofacial implant surgery. Quintessence Publishing, London, pp 74–80

Matsubara T, Suardita K, Ishii M, Sugiyama M, Igarashi A, Oda R, Nishimura M, Saito M, Nakagawa K, Yamanaka K, Miyazaki K, Shimizu M, Bhawal UK, Tsuji K, Nakamura K, Kato Y (2005) Alveolar bone marrow as a cell source for regenerative medicine: differences between alveolar and iliac bone marrow stromal cells. J Bone Miner Res 20:399–409

Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S (2003) SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci USA 13:5807–5812

Nowjack-Raymer RE, Sheiham A (2003) Association of edentulism and diet and nutrition in US adults. J Dent Res 82:123–126

Osugi M, Katagiri W, Yoshimi R, Inukai T, Hibi Dds Phd H, Ueda M (2012) Conditioned media from mesenchymal stem cells enhanced bone regeneration in rat calvarial bone defects. Tissue Eng Part A 18(13–14):1479–1489, Mar 23

Pereira MA, Luiz de Freitas PH, da Rosa TF, Xavier CB (2007) Understanding distraction osteogenesis on the maxillofacial complex: a literature review. J Oral Maxillofac Surg 65:2518–2523

Perin EC, Silva GV (2009) Autologous cell-based therapy for ischemic heart disease: clinical evidence, proposed mechanisms of action, and current limitations. Catheter Cardiovasc Interv 73:281–288, Review

Pierdomenico L, Bonsi L, Calvitti M, Rondelli D, Arpinati M, Chirumbolo G, Becchetti E, Marchionni C, Alviano F, Fossati V, Staffolani N, Franchina M, Grossi A, Bagnara GP (2005) Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation 27:836–842

Raveh J, Sutter F, Hellem S (1987) Surgical procedures for reconstruction of the lower jaw using the titanium-coated hollow-screw reconstruction plate system: bridging of defects. Otolaryngol Clin North Am 20:535–558

Schroeder A, van der Zypen E, Stich H, Sutter F (1981) The reaction of bone, connective tissue and epithelium to endosteal implants with sprayed titanium surfaces. J Oral Maxillofac Surg 9:15–25

Sculean A, Nikolidakis D, Schwarz F (2008) Regeneration of periodontal tissues: combination of barrier membranes and grafting materials – biological foundation and preclinical evidence. A systematic review. J Clin Periodontol 35:106–116

Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364:149–155

Sheiham A, Steele J (2001) Does the condition of the mouth and teeth affect the ability to eat certain foods, nutrient and dietary intake and nutritional status amongst older people? Public Health Nutr 4:797–803

Shi S, Gronthos S (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 8:696–704

Shi S, Bartold PM, Miura M, Seo BM, Robey PG, Gronthos S (2005) The efficacy of mesenchymal stem cells to regenerate and repair dental structures. Orthod Craniofac Res 8:191–199

Smolka W, Iizuka T (2005) Surgical reconstruction of maxilla and midface; Clinical outcome and factors relating to postoperative complications. J Craniomaxillofac Surg 33:1–7

Stewart K, Walsh S, Screen J, Jefferiss CM, Chaainey J, Jordan GR, Beresford JN (1999) Further characterization of cells expressing STRO-1 in cultures of adult human bone marrow stromal cells. J Bone Miner Res 14:1345–1356

Tamari M, Nishino Y, Yamamoto N, Ueda M (2011) Acceleration of wound healing with stem cell-derived growth factors. Oral Craniofac Tissue Eng 1:181–187

The American Academy of Periodontology (1992) Glossary of periodontal terms 38

Torroni A, Gennaro P, Aboh IV, Longo G, Valentini V, Iannetti G (2007) Microvascular reconstruction of the mandible in irradiated patients. J Craniofac Surg 18:1359–1369

Trombelli L, Farina R (2008) Clinical outcomes with bioactive agents alone or in combination with grafting or guided tissue regeneration. J Clin Periodontol 35:117–135

Trulsson U, Engstrand P, Berggren U, Nannmark U, Brånemark P-I (2002) Edentulousness and oral rehabilitation: experiences from the patients' perspective. Eur J Oral Sci 110:417–424

Ueda M, Nishino Y (2010) Cell-based cytokine therapy for skin rejuvenation. J Craniofac Surg 21:1861–1866

Wennerberg A, Albrektsson T (2009) Effects of titanium surface topography on bone integration: a systematic review. Clin Oral Implants Res 20:172–184

Xing Z (2012) Bone tissue engineering: in vitro and in vivo studies of the role of endothelial cells and surface modification of copolymer scaffolds. Ph.D. thesis. Department of Clinical Dentistry, University of Bergen, Norway

Xue Y (2011) Interactions between endothelial and bone marrow stromal cells of relevance to bone tissue engineering. Ph.D. thesis. Department of Clinical Dentistry, University of Bergen, Norway

Yang ZH, Zhang XJ, Dang NN, Ma ZF, Xu L, Wu JJ, Sun YJ, Duan YZ, Lin Z, Jin Y (2009) Apical tooth germ cell-conditioned medium enhances the differentiation of periodontal ligament stem cells into cementum/periodontal ligament-like tissues. J Periodontal Res 44:199–210

Yildirim S, Fu SY, Kim K, Zhou H, Lee CH, Li A, Kim SG, Wang S, Mao JJ (2011) Tooth regeneration: a revolution in stomatology and evolution in regenerative medicine. Int J Oral Sci 3:107–116

Young CS, Terada S, Vacanti JP, Honda M, Bartlett JD, Yelick PC (2002) Tissue engineering of complex tooth structures on biodegradable polymer scaffolds. J Dent Res 81:695–700

Chapter 33
Regenerative Therapies-Trachea

Silvia Baiguera and Paolo Macchiarini

Abstract No preferred treatment, which could offer a functional solution, has been so far developed for patients affected by extensive airway damages. As the field of tissue engineering attempts to develop tracheal replacements, multiple types and combinations of cells, scaffold materials, and/or culture conditions have been used. Interest has been evoked by decellularized natural matrices, which affecting cell proliferation, migration and differentiation, could play an active part in tissue regeneration and remodeling. Using the detergent-enzymatic method, we were able to obtain decellularized human tracheal matrices lacking MHC antigens (bypassing rejection), having structural, mechanical and *in vivo* pro-angiogenic properties similar to that of native airways and supporting *in vivo* recellularization. Starting from these results, we have developed an *in vivo* tissue engineered strategy, based on airway bioengineered grafts combined with autologous stem cells and pharmacological intervention (to boost progenitor cell recruitment commitment), which resulted to be a clinically successful alternative for patients with serious airway disorders.

S. Baiguera, Ph.D.
BIOAIRlab (Laboratory of Biomolecular and Bioengineering Airway), Department of General Thoracic and Regenerative Surgery and Intrathoracic Biotransplantation, University Hospital Careggi, Largo Brambilla 1, Florence 50141, Italy

P. Macchiarini, M.D., Ph.D. (✉)
Department of General Thoracic and Regenerative Surgery, and Intrathoracic Biotransplantation, University Hospital Careggi, Via Olbiate 1, Florence 50141, Italy

University College London, London, UK

Advanced Center for Translational Regenerative Medicine (ACTREM), ENT (CLINTEC), Karolinska Institutet, Alfred Nobel Allé 8, 141 86 Huddinge Stockholm, Sweden
e-mail: paolo.macchiarini@ki.se

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_33,

Abbreviations

DEM	detergent-enzymatic method
EPO	erythropoietin
G-CSF	granulocyte colony-stimulating factor
MSCs	marrow stromal cells
TGF-β	transforming growth factor-β
TNF-α	tumour necrosis factor-α
β-FGF	basic fibroblast growth factor

33.1 Introduction

Any tracheal damage can compromise a wide range of important functions for survival, such as speech, deglutition (swallowing), respiration, mucociliary clearance, and immune protection from inhaled or ingested antigens. In the world, approximately 0.1 every 100,000 persons per year are affected by primary tracheal cancer (Honings et al. 2010), and a smaller, but severely impaired, group of patients are affected by benign disease and trauma linked to non-functioning airways (Nouraei et al. 2007). To date, no conventional solution to treat these patients has been developed.

Regenerative medicine is an interdisciplinary field that "replace or regenerate human cells, tissues or organs, to restore or establish normal function" (Mason and Dunnill 2008) and it has been recently accepted as a useful clinical discipline that ensures and enhances the quality of life in patients undergoing organ reconstructions. Among the different regenerative strategies, tissue engineering is a promising technology that has already provided functional human organ replacements in various clinical settings (Atala et al. 2006). Combining living cells with biocompatible and biodegradable scaffolds, tissue engineering can be applied to the obtainment of an anatomically, physiologically and biomechanically airway replacement which could transform functional outcomes for patients with advanced structural tracheal disorders.

33.2 Tracheal Anatomy and Pathology

The trachea is a fibro-cartilagineous, tubular structure, extending from the cricoids cartilage to the bronchial bifurcation. Structurally, it consists of 18–24 C-shaped cartilaginous rings joined by fibroelastic tissue and closed posteriorly by a membranous muscular structure, named *pars membranacea* (Fig. 33.1a). The trachea functions as an air conduit: the cartilaginous rings prevent collapse during inspiration and widen during expiration, the fibro-elastic tissue prevents over distention, while muscle contraction reduces lumen size facilitating airway clearance. The lateral flexibility of the trachea allows cervical rotation, flexion and extension,

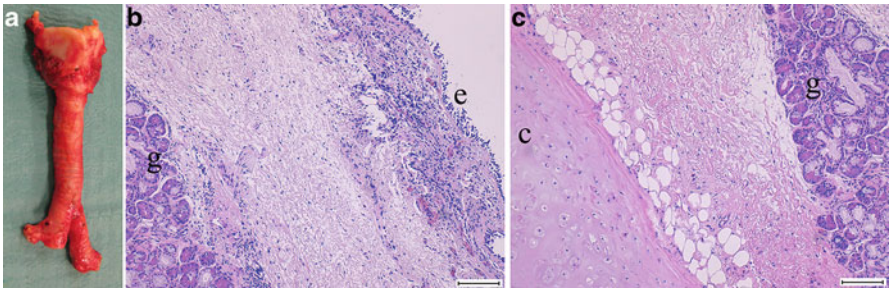


Fig. 33.1 (**a**) Human airway consisting of larynx, trachea and carina. (**b** and **c**) H&E stain of human trachea showing tracheal luminal part covered with a mucosal surface (**b**) and the supporting cartilagineous connective tissue layer beneath the epithelium (**c**). '*c*' cartilaginous part, '*g*' seromucinous glands, '*e*' epithelium (Scale bar = 100 μm)

while maintaining a constant patency for air passage. The tracheal luminal part is covered with a mucosal surface, which serves to protect against infection and aids in mucous clearance, lined with a pseudostratified columnar respiratory epithelium (Fig. 33.1b), containing basal (classical stem cells, which can play a role in airway epithelium homeostasis and regeneration after injury), ciliated, secretory (goblet, serous and Clara cells), neuroendocrine and less well categorized 'indeterminate' or 'intermediate' cells (Mercer et al. 1994; Rock et al. 2010). The supporting connective tissue layer beneath the epithelium, mostly cartilaginous, forms a scaffold hosting blood vessels, nerves and undifferentiated adult stem cells of mesenchymal derivation (which could played a pivotal role in cell repair, regeneration and functional restoration) (Fig. 33.1c) (Okubo et al. 2005; Cardoso and Lü 2006).

A wide spectrum of benign and malignant pathology, most leading to central airway obstruction with subsequent respiratory insufficiency, may afflict the trachea. The exact epidemiology of the tracheal diseases is not perfectly known, being included together with the pathologies affecting the total airway system.

Between benign lesions, tracheomalacia and stenosis (congenital or acquired) are the two most common tracheal anomalies. Tracheomalacia is described as a decreased rigidity of the trachea, due to a structural abnormalities of its wall. In response to variations in intrathoracic pressure or to compression by adjacent intrathoracic structures (esophagus, ascending aorta or aortic arch), tracheomalacia may result in functionally significant interference with air flow and impaired clearance of tracheobronchial secretions. Acquired tracheomalacia may occur following any injury that results in loss of cartilage (such as postintubation injury), chronic external compression or relapsing polychondritis. Stenosis is a fibrotic narrowing of the airway, which can result in severe dyspnea. Although tracheal stenosis is a rare disease, the mortality rates for patients with long-segment tracheal stenosis and atresia (abnormally closed or absent trachea) are nearly 11 and 100%, respectively (Fuchs et al. 2002). There are many causes of stenosis: postintubation injury and tracheostomy are the most common causes of acquired stenosis. Congenital tracheal stenosis, which may involve all or a portion of the tracheal length, is almost always

secondary to a development defect in which the *pars membranacea* is deficient and the wall consists of complete or almost complete cartilaginous rings.

Primary tracheal tumors are the least common neoplastic lesions of the airways, representing the 2% of upper airway tumors (Macchiarini 2006). A majority of primary tumors (more than 90%) in adults are malignant (Macchiarini 2006). By far the most common are adenoid cystic (50%) and squamous cell (20–40%) carcinomas. The adenoid cystic carcinoma is considered to be a slowly growing neoplasma; while squamous cell carcinomas are frequently locally advanced and associated with high incidence of lymph node metastasis (Macchiarini 2006).

In almost all patients with tracheal diseases, resection followed by end-to-end anastomotic reconstruction (surgical joining of two trachea ends to allow air flow from one segment to the other) yields better results and successful outcome than any other treatment. High success rates of over 70% have been reported (Grillo et al. 1995; Omori et al. 2005). However, if a tracheal segment >6 cm long needs to be resected, direct anastomosis is impossible because of the high mechanical tension at the anastomosic site, leading to severe and fatal postoperative complications (Mulliken and Grillo 1968; Grillo 2002). In these cases palliative treatment, such as irradiation, stents and T tubes, are the only solutions (Grillo 2002). Related to primary malignant tracheal tumors, epidemiological studies demonstrated that, due to the difficulties in the definitive diagnosis, most patients present with already advanced local disease and the only possible treatment is the palliative management with stents or neoadjuvant radiotherapy (Gelder and Hetzel 1993; Yang et al. 1997; Licht et al. 2001; Bhattacharyya 2004). For patients non-surgically treated, the 5-year survival pass from 39 to 7% for squamous-cell tumors and from 52 to 33% for adenoid cystic tumors (Licht et al. 2001) and a median survival of less than 12 months has been consistently reported (Chao et al. 1998; Choi 2004).

For these reason, researchers have paid high attention to exploring a solution for tracheal function reconstruction; however, to date, there is no well established good conventional solution. An effective tracheal replacement, displaying anatomical, physiological and biomechanical properties equivalent to normal human airway, could provide an alternative for patients for which standard surgical procedures are not an option.

33.3 Clinical Application of Tracheal Replacement Strategy

In the last century, numerous studies have been made to identify the ideal airway substitute. The various techniques adopted ranges from synthetic stents and prosthetic materials, to autologous/allogenic tissue flaps and patches (Grillo 2002; Osada 2006; Baiguera and Macchiarini 2011). However, none has proven satisfactory for clinical use mainly because of the lack of adequate vascularisation and respiratory epithelium along the lumen (Grillo 2002; Doss et al. 2007). Moreover, it has to be underlined that the trachea is not located in a mesenchymal environment but it is in direct contact with the breathing air, making infection and contamination

more likely to occur. More recently, the enormous efforts in the field of airway replacement allowed the development of strategies which could have relevant clinical applications.

Clinical tracheal allotransplantation have been tried in humans obtaining different results. Fresh or cryopreserved aortic allografts were used, without aid of immunosuppressive therapy, for tracheal, carinal or bronchial replacements (Wurtz et al. 2006, 2010; Wurtz 2010; Martinod et al. 2011): presence of respiratory epithelium and no graft rejection phenomenon were observed. However, fistula development, sparse calcification, progressive graft contraction, stent necessity and/or no evidence of cartilage regeneration were also reported (Wurtz et al. 2006, 2010; Wurtz 2010; Martinod et al. 2011). Non-vital allogenic tracheal grafts resulted suitable only for tracheal patch replacement in the pediatric population, having however the big limitation not to grow with the recipients and resulting in long-term stenosis (Bujia et al. 1991; Elliott et al. 1996; Jacobs et al. 1996, 1999). Recently, the Leuven Tracheal Transplant Group have reported successful tracheal allotransplantation, after withdrawal of immunosuppressive therapy (Delaere et al. 2010). Indirect revascularization was achieved by placing the graft, for 4 months, in the recipient's forearm fascia. During this period, immunosuppressive therapy was necessary, the cartilaginous viability was maintained, while the membranous posterior wall of the allograft underwent avascular necrosis. At the time of transplantation, the tracheal graft (3.5 cm) had viable cartilage fully lined with squamous epithelium, and 1 year after tracheal reconstruction, the patient was satisfied with the outcome and lung functions resulted to be normal (Delaere et al. 2010). Even if clinically successful, the number of operation needed (two major plus eight minor) and the requirement of an 8-month immunosuppression (preventing the use of this strategy in a tumor context) limited the therapeutic potential of this approach.

A regenerative solution providing anatomical restoration of the airway, consisting of a graft with pliability, elasticity, and mucosal lining similar to that of native tissues, which resists stenosis and tolerate implantation, with minimal risk of infection, extrusion, migration or failure, may provide functional airway replacement in a manner superior to that provided by present techniques.

33.4 Regenerative Tracheal Strategy

The tissue engineered approach is normally based on three fundamental components: (i) cells, acting as "seeds" for tissue regeneration, (ii) scaffold, where cells can proliferate and grow, and (iii) regulatory/growth/boosting factors, mediating cell behaviors (Table 33.1). Tissue engineering resulted to be the only technique that seems to offer any real promise, avoiding immunosuppression, for airway replacement and regeneration (Walles et al. 2004a, b; Mertsching et al. 2005).

A variety of regenerative approaches have been proposed for airway replacement, ranging from collagen scaffolds supported by silicones stents, cartilaginous tubes created by *in vitro* culture methods or Marlex mesh tube covered by collagen

Table 33.1 Main components on which the tissue engineered technique is based

	Requirements	Type	Problems
Cells	Non immunogenic	Autologous	Adult primary cell yields and proliferation rates tend to be low
	Highly proliferative	Allogenic	Embryonic stem cells linked to ethical dilemmas and to risks of immunological rejection and tumor formation
	Easy to harvest	Differentiated	
	Able to differentiate into a variety of cell types with specialized functions	Progenitor cells	
		Embryonic/adult stem cells	
		Inducible pluripotent stem cells	
Scaffold	Biocompatible	Natural	Structures have to be tailored to the size and shape required for a particular patient
	Non-immunogenic	Synthetic	Material properties (strength, degradation time, porosity and microstructure) have to be similar to that of native tissue
	Suitable three dimensional template for tissue growth	Biodegradable	Natural matrices related to a limited donor pool
	Sustain/promote cellular growth	Permanent	
	Allow cell adhesion, proliferation and differentiation		
	Facilitate the delivery of vital cell nutrients and waste products		
	Exert mechanical properties similar to that of native tissue		
Factors	Local and/or systemic pharmaceutical intervention to promote autologous progenitor cell mobilization and improve graft integrity	Boosting	Clinical grade
		Recruitment	Possible pro-coagulant effect and hemodynamic changes
		Commitment	Possible thrombembolic risk
			Pro-angiogenesis and indirect tumor promotion

sponge (Teramachi et al. 1997; Kojima et al. 2002; Omori et al. 2005, 2008; Kanzaki et al. 2006; Yamashita et al. 2007); however, none of these strategies resulted adequate for clinical tracheal replacement due to incomplete epithelialisation, with associated stricture, and to a lack of mechanical integrity with consequent tracheomalacic development (Grillo 2002). To provide a biocompatible tracheal substitute with sufficient biological stability, the trachea itself has been hypothesized as the most suitable airway bioprosthesis. This approach relies on the fact that, for tracheal cartilage reconstruction, complex anatomically shaped scaffolds demonstrated to support tissue development better than simple highly modelled designs, the imperfections in the anatomical construct create local niches for increased cell-cell contact and the thinner (nanometric) fibers allow better chondrocyte attachment (Moroni et al. 2007). A tracheal scaffold, conditioned with basic fibroblast growth factor (β-FGF), has been recently implanted in patients affected by stenosis and, 6 months postoperatively, all patients were able to breathe easily (Kanemaru et al. 2010). Even if this new regenerative therapy showed great potential for the treatment of airway diseases, the procedure involved two-staged operations (to enlarge the stenotic region and to implant tracheal scaffold) and β-FGF could not be applied to oncological patients because of tumor recurrence.

Starting from the success of biological scaffolds, derived from decellularized tissues and organs (Ott et al. 2008; Petersen et al. 2010; Uygun et al. 2010; Song et al. 2011), attention has been driven to the possible use of decellularized tracheal matrix to realize functional tracheal replacement. Using a simple and effective procedure, based on osmotic lysis, detergent cell-extraction and DNA digestion (the detergent-enzymatic method) (Meezan et al. 1975), we were able to obtain bioengineered decellularized human tracheal matrices characterized by preserved tissue matrix integrity and biomechanical properties, of sufficient length for clinical application, containing pro-angiogenic factors and supporting *in vivo* recellularization (Fig. 33.2) (Baiguera et al. 2010; Go et al. 2010). This scaffold (seeded with autologous epithelial respiratory cells and mesenchymal stromal cell-derived chondrocytes *via* a bioreactor) allowed to perform the world's first successful transplant of a bioengineered airway (Macchiarini et al. 2008; Baiguera and Macchiarini 2011). Starting from this clinical promising result, we have improved our tissue engineered tracheal approach focusing on the idea that the use of *in vivo* seeded bone marrow stromal cells (MSCs) and of an adequate stimulation (to directly differentiate stromal cells, to mobilize progenitor cells out of the bone marrow and to recruit them at the desired site of transplantation), could allow to obtain a faster tissue repair and remodeling (Bader and Macchiarini 2010).

33.4.1 In Vitro Cell Culture or In Vivo Cell Seeding?

Chondrocytes result essential for the tracheal mechanical properties and the development of a functional cartilage provides the needed support to prevent tracheal collapse. Chondrocyte culture is a well-established procedure, however, de-differentiation

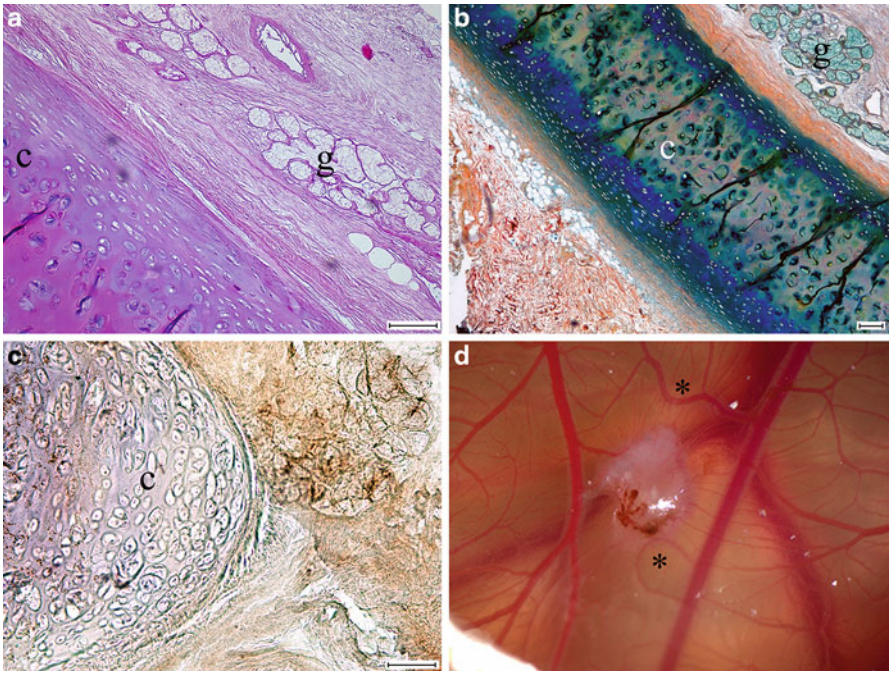


Fig. 33.2 (**a**) H&E stain of decellularized trachea showing the presence of a well preserved and intact extracellular matrix and the absence of cellular elements after 25 detergent-enzymatic cycles. (**b**) Movat pentachromic staining (connective tissue staining) of decellularized matrices. *Yellow* indicates collagen and reticulum fibers; *green to blue green*, ground substance; and *red*, muscle. (**c**) Immunostaining of decellularized tracheal matrix thin sections showing strong immunoreactivity (*brown* staining) against anti-bFGF, a well-known angiogenic factor. (**d**) Representative example of chicken chorioallantoic membrane (CAM) implanted with fragments of decellularized tracheal matrices. Sample was placed on CAM surface of 8-day-old embryos and photographed 4 days later. The sample resulted totally enveloped by CAM vessels and induced a "spoke-wheel" patterns of the new vessels, exerting an influence on vessel network development, as suggested by the looping of vessels toward the matrices (*). '*c*' cartilaginous part, '*g*' seromucinous glands, '*e*' epithelium (Scale bar = 100 μm)

of chondrocytes during culture is a limitation (von der Mark et al. 1977), and *in vitro* engineering of cartilage with valuable quality and functional characteristics is still very challenging. Chondrogenesis is promoted by several growth factors, such as transforming growth factor-β (TGF-β), insulin-like growth factors, fibroblast growth factors, platelet derived growth factor-BB, parathyroid hormone related protein, bone morphogenic proteins and Wnt/β-catenin signalling pathway, and a positive effect on chondrogenic differentiation has been demonstrated by combining various growth factors (Tsutsumi et al. 2001; Mastrogiacomo et al. 2001; Bianchi et al. 2003; Barbero et al. 2003; Solchaga et al. 2005; Kafienah et al. 2006, 2007; Augello and De Bari 2010). However, the optimal combination has not been elucidated so far. TGF-β has been used in different experimental studies, it revealed to be one of the most promising factor for chondrogenic formation (Johnstone et al. 1998; Ronzière et al. 2010)

and the different members of the TGF-β family produce a wide range of effects in different cells and tissues in the body (Cals et al. 2011). Among them, TGF-β3 is emerging as one of the relatively newer isoforms to be discovered and studied. *In vitro* and *in vivo* researches, evaluating the effect of TGF-β3 on chondrogenic differentiation of stem cells, suggested that the implantation of TGF-β3 with chondrocytes in a suitable scaffold may offer a potential biological therapy for cartilaginous regeneration (Tang et al. 2009).

The *in vitro* use of factors poses great concerns because transplanted cells should be clinical grade and animal by-products free for clinical application. Another concern for *in vitro* cultures is that static and mono-dimensional condition cannot mimic the physiological environment. It has been demonstrated that maintaining differentiated chondrocytes in monolayer culture induces a shift of their biosynthetic profile to a fibroblast-like phenotype (Nehrer et al. 1999), with a consequent *in vivo* formation of fibrocartilage instead of hyaline cartilage (Hedbom et al. 1992). On the contrary, chondrocytes cultured in three-dimensional conditions or in macroaggregates remained vital, functional and with a stable phenotype (Walles 2004; Wu et al. 2007). The use of an *in vitro* bioreactor or of an *in vivo* subcutaneous pre-conditioning has been also evaluated. However, all these approaches requires time and money, introduces regulatory challenges and are related to bacterial contamination risks.

Recently, it has been suggested that a suitable regenerative strategy could be to avoid any *in vitro* cellular or construct manipulation and to directly implant the construct together with a pharmacological mix of specific growth factors to induce *in vivo* physiological regeneration (Bader and Macchiarini 2010). In this approach, the replacement is designed to take place in the body after implantation and thus is referred to as *in situ* or *in vivo* tissue engineering (Bader and Macchiarini 2010). This concept has been recently used by Nakamura and colleagues (2009), which, seeding intraoperatively a synthetic polypropylene scaffold with autologous bone marrow MSCs, were able to *in vivo* rebuild a trachea-like organ, with a good epithelial lining. The positive results obtained suggested that starting from in vivo seeded bone marrow stromal cells and using the body of the recipient patient as a biological, natural bioreactor, the *in vivo* tissue engineering approach may facilitate tracheal reconstruction.

Based on these idea, we improved our strategy by seeding the decellularized human tracheal graft with a mixture of mononuclear cells, MSCs and patient own blood directly after isolation in the surgery theatre, avoiding therefore any *in vitro* culture step.

33.4.2 Pharmacological Therapy

Direct intraoperative application of cells is an interesting and promising approach, however we suppose that graft survival could be supported by the activation of the endogenous repair system. A proper pharmaceutical intervention, by which mobilize

endogenous stem and progenitor cells, could result then essential to promote a successful long-term graft regeneration.

It has been demonstrated that growth factors and cytokines, produced by injured and inflammatory cells, are able to activate, mobilize and attract different types of progenitor cells (Singer and Caplan 2011; Marquez-Curtis et al. 2011), and cytokines, in particular, have been accepted as "standard" agents to mobilize progenitor stromal cells (like hematopoietic) from bone marrow to peripheral blood. To date, the granulocyte colony-stimulating factor (G-CSF) has become a standard agent in current clinical practice because it has been shown to mobilize significantly more peripheral blood stromal cells and resulted less toxic than other cytokines (Takeyama and Ohto 2004). Moreover, addition of erythropoietin (EPO), a cytokine mainly active on erythropoiesis, to G-CSF has been demonstrated to increase peripheral blood stromal cell mobilization and resulted in significantly higher yields of $CD34^+$ cells (Takeyama and Ohto 2004). The EPO influences in the peripheral circulation is well described and it has been observed in different tissues (Brines and Cerami 2008; Jungebluth et al. 2012). It has been determined that, in the presence of an inflammatory region, local EPO production is repressed by high tumour necrosis factor-α (TNF-α) levels, while the EPO receptor becomes up-regulated by pro-inflammatory cytokines, resulting a potential target region for pharmaceutical intervention (Brines and Cerami 2008; Brines 2010). Moreover, it has been demonstrated that EPO, by binding to β–common receptor (which does not have any contribution to the erythropoiesis but significant impact on tissue protection), induces a tissue protective effect in different tissue types and diseased conditions, particularly in ischemic settings of the lung, liver, heart, chronic skin ulcera or burn, trauma, cytokine-related acute injuries (Brines and Cerami 2008). Based on these data and to counteract apoptotic events within the trachea graft, which occur due to surgery induced injury and to a lack in vascularization within the newly transplanted scaffold, we decided to submit transplanted patients to a regenerative therapy, by giving post-operative injections of G-CSF and EPO in order to increase construct regeneration.

G-CSF is almost an acceptable agent for peripheral blood cell mobilization, but it has obvious toxicities, such as bone pain, fever, reversible elevations of alkaline phosphatase, lactate dehydrogenase, asparate aminotransferase and uric acid and rare case of thrombosis, myocardial infarction, adult respiratory distress syndrome, and splenic rupture (Takeyama and Ohto 2004). Also EPO treatment is related with negative side effects, such as hemodynamic changes and thrombembolic risks. Close careful monitoring of the patient's condition, especially hematocrit, during regenerative therapy administration is then necessary for detecting early signs of complications.

33.5 *In Vivo* Regenerative Tracheal Strategy

The new developed *in vivo* tracheal strategy is based on decellularized human tracheas intraoperatively seeded with autologous bone marrow MSCs, for the external, and respiratory epithelial cells, for the internal, tracheal surface (completely avoiding

in vitro cell culturing and using the patient's own body as a "living natural" bioreactor), and intraoperatively conditioned with differentiative (TGF-β3, to allow stem cell's differentiation into cartilage-tissue), growing (G-CSF, to promote stem cell mobilization and recruitment to the injured sites), and 'boosting' (EPO, to activate and stimulate stem cell recruitment and wound healing) factors (Table 33.2) (Kalathur et al. 2010). After implantation, the therapy is intensified by giving post-operative systemic injections of G-CSF and EPO at 'regenerative' dose levels every other day for 14 days.

This *in vivo* regenerative approach has been so far adopted in five patients with benign tracheal diseases and in two patients with primary tracheal cancers, involving the entire trachea (http://abcnews.go.com/Health/Health/successful-stem-cell-trachea-transplant/story?id=11308383). The cytofluorimetric analysis of peripheral blood cell mobilization showed a steady increase in the number of the hematopoietic progenitor cells ($CD34^+$) during the peri-transplantation period, suggesting that peripheral blood (hematopoietic) stromal cell recruitment may play a vital role in the overall success of the replacement airway. To date, the new *in vivo* engineered transplanted tracheas are able to support themselves, have proved to possess a good epithelial coating, immediate vascularization and, upon all, a constantly wide open lumen for air passage.

These early successful results demonstrate that our strategy, based on optimally bioengineered materials combined with autologous cells and pharmacological intervention, to boost progenitor cell recruitment and thereby promoting tissue formation and regeneration *in situ*, could provide a therapeutic option and eventually a better alternative solution for patients with serious clinical tracheal disorders.

Besides these clinical successful outcomes, this approach has some limitations, like a relatively long period for the decellularization process (15–20 days), the risks for altering long-term natural matrix mechanical properties and for bacterial contamination during the *in vitro* natural graft manipulation and, most importantly, the absolute requirement of obtaining a donor organ and the reliance on donor tissues. A suitable approach to obtain a long-term preservation is one of the methods to help solve the limitation related to donor tissue shortage. We have reported that the immunological and mechanical characteristics of the acellular pig matrices remained unaffected by a 2-month storage in phosphate-buffered saline solution (containing 1% antibiotic and antimycotic, at 4 °C) (Jungebluth et al. 2009), however longer preservation periods have not been evaluated. Very recently, we have demonstrated that after 1-year storage, human decellularized tracheas were characterized by a general damage of the extracellular matrix histoarchitecture, particularly collagenous and elastic fiber structure, resulting in decreased mechanical and angiogenic properties. Considering that the quality of the structural matrix at implantation may predetermine durability or failure of preserved graft, human decellularized tracheas stored for 1 year in phosphate-buffered saline solution at 4 °C, would not meet the demands for a tissue engineering matrix and likely would not yield a suitable graft for lifelong implantation. Moreover, the degradation phenomenon observed *in vitro* may be further enhanced *in vivo*, having clinical relevance for tissues that will be

Table 33.2 *In vitro* and *in vivo* bioengineering tracheal procedures

		Procedure		Timing	
		In vitro	*In vivo*	*In vitro*	*In vivo*
Scaffold		Human donor trachea decellularized by DEM	Human donor trachea decellularized by improved DEM	2 months	3 weeks
Cells	External surface	Autologous bone marrow MSCs *in vitro* differentiated into chondrocytes	Autologous bone marrow MSCs	3 months	2 h
	Internal surface	*In vitro* cultured autologous respiratory epithelial cells	Autologous tracheal (or nasal) respiratory epithelial cells	3 months	15 min
Bioreactor		Double-chamber bioreactor	Patient body Growthing and boosting factors		
Construct obtainment		Cells seeded onto the matrix and *in vitro* dynamically cultured	Cellular conditioning, boosting and differentiative factor injection performed immediately before transplant.	96 h	Intraoperative

DEM detergent-enzymatic method, *MSCs* mesenchymal stem cells

transplanted long-term and this should be carefully evaluated in pre-clinical settings (Baiguera et al. 2012).

Another solution could be represented by the development of a synthetic construct, that mimics the structural and mechanical tracheal properties. Very recently, based on our experience on human tracheal matrix characteristics (Baiguera et al. 2010) and using a nanocomposite polymeric material, we were able to obtain a tailored-made artificial tracheal scaffold with physical and mechanical properties similar to native tissue. The artificial scaffold, *ex-vivo* seeded with autologous progenitor cells (*via* bioreactor) and conditioned with pharmacology therapy, has been successfully implanted into a patient affected by primary recurrent tracheobronchial tumour (Jungebluth et al. 2011). After three postoperative months, the nanocomposite was lined with a well-developed healthy mucosa and no distal ischemic necrosis was observable. Moreover, cellular biochemistry analyses have provided new insight into the mechanisms by which the utilized regenerative therapy contribute to cell mobilization, differentiation and ultrastructural organization of the fully engrafted tracheobronchial construct (Jungebluth et al. 2011). The successful overall clinical outcome of this first-in-man bioengineered artificial tracheobronchial transplantation provides ongoing proof of the viability of this approach, where a cell-seeded synthetic graft is fabricated to patient-specific anatomical requirements and incubated to maturity within the environment of a bioreactor.

33.6 Conclusions

Successful bioengineering airway transplants have been obtained, within the past 3 years, thanks to a thorough interdisciplinary work. Patients transplanted can now breathe normally without any immunosuppression, and the application of the *in vivo* tissue engineering technique in cancer patients, although requiring further follow-up, is very promising and may be curative. These early successful results demonstrate that the *in vivo* tissue engineered strategy, based on optimally bioengineered materials combined with autologous cells and pharmacological intervention, to boost progenitor cells, could provide a therapeutic option and eventually a cure for patients with serious clinical airway disorders. However, important questions, such as the evaluation of the long-term biomechanical properties of the bioengineered grafts, the optimization of tracheal synthetic graft and of bioreactor design, function and fate of the seeded cells in tissue regeneration and mechanisms of angiogenesis, remain to be answered before a full clinical trial accreditation may be obtained.

References

Atala A, Bauer SB, Soker S, Yoo JJ, Retik AB (2006) Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet 367:1241–1246

Augello A, De Bari C (2010) The regulation of differentiation in mesenchymal stem cells. Hum Gene Ther 21(10):1226–1238
Bader A, Macchiarini P (2010) Moving towards in situ tracheal regeneration: the bionic tissue engineered transplantation approach. J Cell Mol Med 14(7):1877–1889
Baiguera S, Macchiarini P (2011) Trachea. In: Steinhoff G (ed) Regenerative medicine – from protocol to patient, 1st edn. Springer, London, pp 691–711
Baiguera S, Jungebluth P, Burns A, Mavilia C, Haag J, De Coppi P, Macchiarini P (2010) Tissue engineered human tracheas for in vivo implantation. Biomaterials 31(34):8931–8938
Baiguera S, Del Gaudio C, Jaus MO, Polizzi L, Gonfiotti A, Comin CE, Bianco A, Ribatti D, Taylor DA, Macchiarini P (2012) Long-term changes to in vitro preserved bioengineered human trachea and their implications for decellularized tissues. Biomaterials 33(14):3662–3672. doi:10.1016/j.biomaterials.2012.01.064
Barbero A, Ploegert S, Heberer M, Martin I (2003) Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum 48:1315–1325
Bhattacharyya N (2004) Contemporary staging and prognosis for primary tracheal malignancies: a population-based analysis. Otolaryngol Head Neck Surg 131:639–642
Bianchi G, Banfi A, Mastrogiacomo M, Notaro R, Luzzatto L, Cancedda R, Quarto R (2003) Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. Exp Cell Res 287:98–105
Brines M (2010) The therapeutic potential of erythropoiesis-stimulating agents for tissue protection: a tale of two receptors. Blood Purif 29(2):86–92
Brines M, Cerami A (2008) Erythropoietin-mediated tissue protection: reducing collateral damage from the primary injury response. J Intern Med 264(5):405–432
Bujia J, Pitzke P, Krombach F, Hammer C, Wilmes E, Herberhold C, Kastenbauer E (1991) Immunological behavior of preserved human tracheal allografts: immunological monitoring of a human tracheal recipient. Clin Transplant 5:376–380
Cals FL, Hellingman CA, Koevoet W, Baatenburg de Jong RJ, van Osch GJ (2011) Effects of transforming growth factor-β subtypes on in vitro cartilage production and mineralization of human bone marrow stromal-derived mesenchymal stem cells. J Tissue Eng Regen Med 6(1):68–76. doi:10.1002/term.399
Cardoso WV, Lü J (2006) Regulation of early lung morphogenesis: questions, facts and controversies. Development 133(9):1611–1624
Chao MW, Smith JG, Laidlaw C, Joon DL, Ball D (1998) Results of treating primary tumors of the trachea with radiotherapy. Int J Radiat Oncol Biol Phys 41:779–785
Choi NC (2004) Radiation therapy in the management of tracheal cancer. In: Grillo HC (ed) Surgery of the trachea and bronchi. BC Decker, Hamilton/London, pp 791–802
Delaere P, Vranckx J, Verleden G, De Leyn P, Van Raemdonck D, Leuven Tracheal Transplant Group (2010) Tracheal allotransplantation after withdrawal of immunosuppressive therapy. N Engl J Med 362:138–145
Doss AE, Dunn SS, Kucera KA, Clemson LA, Zwischenberger JB (2007) Tracheal replacements: part 2. ASAIO J 53(5):631–639
Elliott MJ, Haw MP, Jacobs JP, Bailey CM, Evans JN, Herberhold C (1996) Tracheal reconstruction in children using cadaveric homograft trachea. Eur J Cardiothorac Surg 10:707–712
Fuchs JR, Terada S, Ochoa ER, Vacanti JP, Fauza DO (2002) Fetal tissue engineering: in utero tracheal augmentation in an ovine model. J Pediatr Surg 37(7):1000–1006
Gelder CM, Hetzel MR (1993) Primary tracheal tumours: a national survey. Thorax 48:688–692
Go T, Jungebluth P, Baiguera S, Asnaghi A, Martorell J, Ostertag H, Mantero S, Birchall M, Bader A, Macchiarini P (2010) Both epithelial cells and mesenchymal stem cell-derived chondrocytes contribute to the survival of tissue-engineered airway transplants in pigs. J Thorac Cardiovasc Surg 139(2):437–443
Grillo HC (2002) Tracheal replacement: a critical review. Ann Thorac Surg 73(6):1995–2004
Grillo HC, Donahue DM, Mathisen DJ, Wain JC, Wright CD (1995) Postintubation tracheal stenosis. Treatment and result. J Thorac Cardiovasc Surg 109(3):486–492

Hedbom E, Antonsson P, Hjerpe A, Aeschlimann D, Paulsson M, Rosa-Pimentel E, Sommarin Y, Wendel M, Oldberg A, Heinegård D (1992) Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. J Biol Chem 267:6132–6136

Honings J, Gaissert HA, van der Heijden HF, Verhagen AF, Kaanders JH, Marres HA (2010) Clinical aspects and treatment of primary tracheal malignancies. Acta Otolaryngol 130:763–772

http://abcnews.go.com/Health/Health/successful-stem-cell-trachea-transplant/story?id=11308383

Jacobs JP, Elliott MJ, Haw MP, Bailey CM, Herberhold C (1996) Pediatric tracheal homograft reconstruction: a novel approach to complex tracheal stenosis in children. J Thorac Cardiovasc Surg 112:1549–1560

Jacobs JP, Quintessenza JA, Andrews T, Burke RP, Spektor Z, Delius RE, Smith RJ, Elliott MJ, Herberhold C (1999) Tracheal allograft reconstruction: the total North American and worldwide pediatric experiences. Ann Thorac Surg 68:1043–1052

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238(1):265–272

Jungebluth P, Go T, Asnaghi A, Bellini S, Martorell J, Calore C, Urbani L, Ostertag H, Mantero S, Conconi MT, Macchiarini P (2009) Structural and morphologic evaluation of a novel detergent-enzymatic tissue-engineered tracheal tubular matrix. J Thorac Cardiovasc Surg 138(3):586–593

Jungebluth P, Alici E, Baiguera S, Blanc KL, Blomberg P, Bozoky B, Crowley C, Einarsson O, Grinnemo KH, Gudbjartsson T, Guyader SL, Henriksson G, Hermanson O, Juto JE, Leidner B, Lilja T, Liska J, Luedde T, Lundin V, Moll G, Nilsson B, Roderburg C, Stromblad S, Sutlu T, Teixeira AI, Watz E, Seifalian A, Macchiarini P (2011) Tracheobronchial transplantation using a stem cell-seeded bioartificial nanocomposite. Lancet 378(9808):1997–2004

Jungebluth P, Moll G, Baiguera S, Macchiarini P (2012) Tissue engineered airway: a regenerative solution. Clin Pharmacol Ther 91(1):81–93

Kafienah W, Mistry S, Williams C, Hollander AP (2006) Nucleostemin is a marker of proliferating stromal stem cells in adult human bone marrow. Stem Cells 24:1113–1120

Kafienah W, Mistry S, Dickinson SC, Sims TJ, Learmonth I, Hollander AP (2007) Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients. Arthritis Rheum 56:177–187

Kalathur M, Baiguera S, Macchiarini P (2010) Translating tissue-engineered tracheal replacement from bench to bedside. Cell Mol Life Sci 67(24):4185–4196

Kanemaru S, Hirano S, Umeda H, Yamashita M, Suehiro A, Nakamura T, Maetani T, Omori K, Ito J (2010) A tissue-engineering approach for stenosis of the trachea and/or cricoid. Acta Otolaryngol Suppl 563:79–83

Kanzaki M, Yamato M, Hatakeyama H, Kohno C, Yang J, Umemoto T, Kikuchi A, Okano T, Onuki T (2006) Tissue engineered epithelial cell sheets for the creation of a bioartificial trachea. Tissue Eng 12:1275–1283

Kojima K, Bonassar LJ, Roy AK, Vacanti CA, Cortiella J (2002) Autologous tissue-engineered trachea with sheep nasal chondrocytes. J Thorac Cardiovasc Surg 123:1177–1184

Licht PB, Friis S, Pettersson G (2001) Tracheal cancer in Denmark: a nationwide study. Eur J Cardiothorac Surg 19:339–3345

Macchiarini P (2006) Primary tracheal tumours. Lancet Oncol 7:83–91

Macchiarini P, Jungebluth P, Go T, Asnaghi MA, Rees LE, Cogan TA, Dodson A, Martorell J, Bellini S, Parnigotto PP, Dickinson SC, Hollander AP, Mantero S, Conconi MT, Birchall MA (2008) Clinical transplantation of a tissue-engineered airway. Lancet 372:2023–2030

Marquez-Curtis LA, Turner AR, Sridharan S, Ratajczak MZ, Janowska-Wieczorek A (2011) The ins and outs of hematopoietic stem cells: studies to improve transplantation outcomes. Stem Cell Rev 7:590–607

Martinod E, Radu DM, Chouahnia K, Seguin A, Fialaire-Legendre A, Brillet PY, Destable MD, Sebbane G, Beloucif S, Valeyre D, Baillard C, Carpentier A (2011) Human transplantation of a biologic airway substitute in conservative lung cancer surgery. Ann Thorac Surg 91(3):837–842

Mason C, Dunnill P (2008) A brief definition of regenerative medicine. Regen Med 3(1):1–5

Mastrogiacomo M, Cancedda R, Quarto R (2001) Effect of different growth factors on the chondrogenic potential of human bone marrow stromal cells. Osteoarthritis Cartilage 9(Suppl A):S36–S40

Meezan E, Hjelle JT, Brendel K (1975) A simple, versatile, nondisruptive method for the isolation of morphologically and chemically pure basement membranes from several tissues. Life Sci 17:1721–1732

Mercer RR, Russell ML, Roggli VL, Crapo JD (1994) Cell number and distribution in human and rat airways. Am J Respir Cell Mol Biol 10:613–624

Mertsching H, Walles T, Hofmann M, Schanz J, Knapp WH (2005) Engineering of a vascularized scaffold for artificial tissue and organ generation. Biomaterials 33:6610–6617

Moroni L, Curti M, Welti M, Korom S, Weder W, de Wijn JR, van Blitterswijk CA (2007) Anatomical 3D fiber-deposited scaffolds for tissue engineering: designing a neotrachea. Tissue Eng 13:2483–2493

Mulliken JB, Grillo HC (1968) The limits of tracheal resection with primary anastomosis: further anatomical studies in man. J Thorac Cardiovasc Surg 55(3):418–421

Nakamura T, Sato T, Araki M (2009) In situ tissue engineering for tracheal reconstruction using a luminar remodeling type of artificial trachea. J Thorac Cardiovasc Surg 138(4):811–819

Nehrer S, Spector M, Minas T (1999) Histologic analysis of tissue after failed cartilage repair procedures. Clin Orthop Relat Res 365:149–162

Nouraei SAR, Nouraei SM, Howard DJ, Sandhu GS (2007) Estimating the population incidence of adult post-intubation laryngotracheal stenosis. Clin Otolaryngol 32:407–409

Okubo T, Knoepfler PS, Eisenman RN, Hogan BL (2005) Nmyc plays an essential role during lung development as a dosage-sensitive regulator of progenitor cell proliferation and differentiation. Development 132(6):1363–1374

Omori K, Nakamura T, Kanemaru S, Asato R, Yamashita M, Tanaka S, Magrufov A, Ito J, Shimizu Y (2005) Regenerative medicine of the trachea: the first human case. Ann Otol Rhinol Laryngol 114(6):429–433

Omori K, Tada Y, Suzuki T, Nomoto Y, Matsuzuka T, Kobayashi K, Nakamura T, Kanemaru S, Yamashita M, Asato R (2008) Clinical application of in situ tissue engineering using a scaffolding technique for reconstruction of the larynx and trachea. Ann Otol Rhinol Laryngol 117(9):673–678

Osada H (2006) Artificial trachea. J Broncho 13:39–43

Ott HC, Matthiesen TS, Goh SK, Black LD, Kren SM, Netoff TI, Taylor DA (2008) Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med 14:213–221

Petersen TH, Calle EA, Zhao L, Lee EJ, Gui L, Raredon MB, Gavrilov K, Yi T, Zhuang ZW, Breuer C, Herzog E, Niklason LE (2010) Tissue-engineered lungs for in vivo implantation. Science 329:538–541

Rock JR, Randell SH, Hogan BL (2010) Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. Dis Model Mech 3(9–10):545–556

Ronzière MC, Perrier E, Mallein-Gerin F, Freyria AM (2010) Chondrogenic potential of bone marrow- and adipose tissue-derived adult human mesenchymal stem cells. Biomed Mater Eng 20(3):145–158

Singer NG, Caplan AI (2011) Mesenchymal stem cells: mechanisms of inflammation. Annu Rev Pathol 6:457–478

Solchaga LA, Penick K, Porter JD, Goldberg VM, Caplan AI, Welter JF (2005) FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. J Cell Physiol 203:398–409

Song JJ, Kim SS, Liu Z, Madsen JC, Mathisen DJ, Vacanti JP, Ott HC (2011) Enhanced in vivo function of bioartificial lungs in rats. Ann Thorac Surg 92:998–1005

Takeyama K, Ohto H (2004) PBSC mobilization. Transfus Apher Sci 31(3):233–243

Tang QO, Shakib K, Heliotis M, Tsiridis E, Mantalaris A, Ripamonti U, Tsiridis E (2009) TGF-beta3: a potential biological therapy for enhancing chondrogenesis. Expert Opin Biol Ther 9(6):689–701

Teramachi M, Nakamura T, Yamamoto Y, Kiyotani T, Takimoto Y, Shimizu Y (1997) Porous-type tracheal prosthesis sealed with collagen sponge. Ann Thorac Surg 64:965–969

Tsutsumi S, Shimazu A, Miyazaki K, Pan H, Koike C, Yoshida E, Takagishi K, Kato Y (2001) Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. Biochem Biophys Res Commun 288:413–419

Uygun BE, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA, Shulman C, Milwid J, Kobayashi N, Tilles A, Berthiaume F, Hertl M, Nahmias Y, Yarmush ML, Uygun K et al (2010) Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat Med 16:814–820

von der Mark K, Gauss V, von der Mark H, Müller P (1977) Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature 267(5611):531–532

Walles T (2004) Bioartificial tracheal grafts: can tissue engineering keep its promise? Expert Rev Med Devices 1(2):241–250

Walles T, Giere B, Hofmann M, Schanz J, Hofmann F, Mertsching H, Macchiarini P (2004a) Experimental generation of a tissue-engineered functional and vascularized trachea. J Thorac Cardiovasc Surg 128(6):900–906

Walles T, Giere B, Macchiarini P, Mertsching H (2004b) Expansion of chondrocytes in a three-dimensional matrix for tracheal tissue engineering. Ann Thorac Surg 78:444–448

Wu W, Cheng X, Zhao Y, Chen F, Feng X, Mao T (2007) Tissue engineering of trachea-like cartilage grafts by using chondrocyte macroaggregate: experimental study in rabbits. Artif Organs 31(11):826–834

Wurtz A (2010) Tracheal replacement with banked cryopreserved aortic allograft. Ann Thorac Surg 89(6):2072

Wurtz A, Porte H, Conti M, Desbordes J, Copin MC, Azorin J, Martinod E, Marquette CH (2006) Tracheal replacement with aortic allografts. N Engl J Med 355(18):1938–1940

Wurtz A, Porte H, Conti M, Dusson C, Desbordes J, Copin MC, Marquette CH (2010) Surgical technique and results of tracheal and carinal replacement with aortic allografts for salivary gland-type carcinoma. J Thorac Cardiovasc Surg 140(2):387–393

Yamashita M, Kanemaru S, Hirano S, Magrufov A, Tamaki H, Tamura Y, Kishimoto M, Omori K, Nakamura T, Ito J (2007) Tracheal regeneration after partial resection: a tissue engineering approach. Laryngoscope 117:497–502

Yang KY, Chen YM, Huang MH, Perng RP (1997) Revisit of primary malignant neoplasms of the trachea: clinical characteristics and survival analysis. Jpn J Clin Oncol 27:305–309

Chapter 34
Lung

Lavinia Iuliana Ionescu and Bernard Thébaud

Abstract Recent discoveries in stem cell biology have generated excitement about the possibility of harnessing stem cells for repair and regeneration of lung diseases. Although initial emphasis was on engraftment of stem cells in lung, more recent studies demonstrate that mesenchymal stem cells (MSCs) can modulate local inflammatory and immune responses in experimental lung disease models including acute lung injury and pulmonary fibrosis via a paracrine activity. Endothelial progenitor cells (EPCs) also seem to contribute to lung repair and are used in clinical trials for the treatment of pulmonary hypertension. The ability to produce stem cells by induced pluripotency may relieve many ethical concerns related to the use of embryonic stem cells and may open the way to large-scale production and evaluation of pluripotent stem cells for lung regeneration and repair. The aim of this review is to provide a summary of the recent progress made in the field of lung regeneration using different approaches including stem cell-based therapy and lung tissue bioengineering.

L.I. Ionescu, M.D., Ph.D.
Department of Physiology, 3032 Katz Group Centre for Pharmacy and Health Research, University of Alberta, Edmonton, AB T6G 2S2, Canada

B. Thébaud, M.D., Ph.D. (✉)
Professor of Pediatrics, Department of Pediatrics, Children's Hospital of Eastern Ontario

Senior Scientist, Ottawa Hospital Research Institute, Sprott Center for Stem Cell Research

Department of Cellular and Molecular Medicine, University of Ottawa,
501 Smyth Road, Ottawa, ON K1H 8L6, Canada
e-mail: bthebaud@ohri.ca

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_34,

34.1 Introduction

The respiratory system supports the vital function of breathing. It can be viewed as the interface between the oxygen-rich environment and the carbon dioxide-producing living organism. The failure of the lungs to complete their function is immediately life-threatening.

From a functional and anatomical viewpoint, the respiratory system comprises two compartments: the conducting airways (nasal cavity, pharynx, larynx, trachea, bronchi and bronchioles) and the gas-exchanging airways (respiratory bronchioles and the saccular-alveolar compartment, where alveolar walls come in close contact with capillary walls in order to facilitate the exchange of oxygen and carbon dioxide). Lung injury can occur at any of these levels leading to impairment of breathing function, which can be reversible or irreversible. Obstructive respiratory diseases, such as asthma and chronic bronchitis are caused by damage at the airway level, which limits airflow, whereas restrictive pulmonary diseases, such as lung fibrosis, acute respiratory distress syndrome (ARDS), sarcoidosis are determined by inflammatory processes in the lung interstitium, leading to reduced lung compliance with limitation of lung expansion. Although the advancements of biomedical research over the past decades have brought novel therapeutic approaches for respiratory disorders, many lung diseases, such as chronic lung disease of prematurity (or bronchopulmonary dysplasia, BPD), chronic obstructive pulmonary disease (COPD) and cystic fibrosis are still lacking efficient treatments. According to the WHO World Health Report 2000, lung diseases contribute to a total of 17.4% of deaths and 13.3% of disability-adjusted life years (DALY) worldwide (World Health Organization, World Health Report 2000). These facts highlight the absolute necessity to study the potential applicability of recent developments in the field of regenerative medicine as therapeutic options for lung diseases.

34.2 Lung Development and Regeneration

The current view is that *in situ* regeneration can occur in parenchymal organs following injury as long as the organ framework has been sufficiently preserved. Also, it is believed that the regeneration principles would normally follow evolutionary principles and likely recapitulate ontogeny (Warburton et al. 2001; Cardoso and Whitsett 2008). The intrauterine development of the lung has traditionally been subdivided in five overlapping stages, on the basis of gross histological features (Burri 2006). The respiratory system starts forming as early as the third week of gestation as an outpouching of the primitive forgut bifurcating into the two main stem bronchi (***embryonic stage***). During the ***pseudo-glandular stage***, rudimentary bronchi divide by dichotomous branching; these tubular structures are lined by columnar epithelium surrounded by mesenchymal tissue. The ***canalicular stage*** is characterized by the bifurcation of the last generations of distal bronchi. In this stage there is also capillary invasion and differentiation of the air space epithelium

into type II cells (responsible for surfactant production) and type I cells (which form the thin air-blood barriers). During the ***saccular stage*** the peripheral air spaces expand in length and width, and, at the expense of the intervening mesenchyme, to form saccules at about 36 weeks. Alveolarization, the final stage of lung development, begins in the near-term lung prior to birth but primarily occurs postnatally, during the first 2–3 years of life, and may continue at a slower rate beyond childhood. The alveolus is the definitive gas-exchanging unit- of the lung. Alveoli are, in part, formed by subdivision (*septation*) of the saccules. Septation involves budding of septal crests, which is followed by elongation of the septal walls to form individual alveoli. Septation increases the gas-exchange surface area, without a proportionate increase of lung volume (i.e. alveoli have a larger surface/volume ratio than saccules). ***Microvascular maturation***, the final important step in lung development, follows and partly overlaps the alveolar stage. The capillaries – which form double capillary layers in the immature gas-exchange region – remodel to form a single capillary layer. The thickness of the alveolar wall decreases about 20% and the distance between alveolar gas and capillary blood diminishes about 25%. Morphometric studies show that from birth to adulthood, the alveolar and capillary surface areas expand about 20-fold and the capillary volume 35-fold.

While the histological changes are well described (Zeltner and Burri 1987; Kitaoka et al. 1996; Maeda et al. 2007), much more needs to be learned about the mechanisms that regulate normal lung development in order to harness these processes for therapeutic purposes (Warburton et al. 2001). This is particularly relevant for the perinatal care of extremely premature infants who are born at the late canalicular stage before the completion of the alveolar stage. The immaturity of the lungs, together with the ventilator support required places these infants at risk of developing BPD, which may lead to an irreversible arrest in alveolar development and impaired lung function beyond childhood (Wong et al. 2008).

The lack of efficient therapies that would prevent or repair lung damage in diseases such as BPD, cystic fibrosis, ARDS, emphysema, or pulmonary fibrosis together with recent findings that suggest therapeutic benefits of stem cell-based approaches in animal models of lung disease, have turned the use of stem cells into a new and exciting avenue in lung regenerative medicine (Lane et al. 2007).

34.3 Stem Cells

Stem cells are defined as cells that have clonogenic and self-renewal potential and are able to differentiate along multiple cell lineages. The size, shape and cellular compartments of the adult organs are determined by embryonic and fetal stem/progenitor cell behavior. Traditionally, stem cells are categorized based on their origin and differentiation potential into embryonic and adult (postnatal) stem cells. **Embryonic stem cells (ES)** are isolated from the inner mass of the trophoblast and are characterized by their ability to differentiate along multiple cell lineages originating in all three germ layers (pluripotency), whereas the differentiation potential

of **adult stem cells** (multipotent or, for progenitor cells, oligo- or unipotent) has been considered to be limited by their germ layer origin. However, recent studies are challenging this paradigm, as stem cells derived from bone marrow, either classically considered to be partially committed to the hematopoietic or mesenchymal lineages, have been shown to cross lineage boundaries and transdifferentiate along lineages derived from a different germ layer.

To date, ES cells are better characterized than adult stem cells. Yet, the lineage relationship between embryonic and adult stem/progenitor cells has not been clearly described.

One of the defining features of stem cells is their ability to divide either symmetrically, generating two identical "daughter" cells or asymmetrically, giving rise to an identical "daughter" stem cell and a more specialized, lineage-committed progenitor cell (Rawlins 2008) that lacks the self-renewal ability but possesses a higher proliferation rate compared to its parent stem cell. It becomes obvious that a tight regulatory control of the balance between symmetrical and asymmetrical division, as well as the proliferation rate of these cells, is critical for organ development and homeostasis. For instance, it has been proposed that at each stage of lung development the stem cells divide mostly in an asymmetrical fashion, leaving the specialized progenitor behind as the identical "daughter" cell moves distally with the budding lung tips.

34.4 Resident Lung Stem/Progenitor Cells

At birth, the normally developed lung will comprise more than 40 cell types that originate in both endoderm and mesoderm layers: basal cells, ciliated cells, secretory (goblet, Clara, nonciliated) cells, type I and type II alveolar epithelial cells (pneumocytes), airway smooth muscle cells, capillary endothelial cells, fibroblasts, alveolar macrophages, mast cells, dendritic cells. In healthy adults, lung cellular homeostasis is viewed as a slow process compared to highly proliferating tissues such as the bone marrow, intestine or skin, which makes it more difficult to study lung resident stem/progenitor cells. However, it is widely accepted that stem/progenitor cells contribute to maintenance of lung cell populations and there is evidence that stem cell proliferation rate in the lung increases dramatically following injury and that the type and amplitude of injury also determines the intensity, duration and type of cellular response (reviewed by Gomperts and Strieter 2007).

Current approaches in lung regenerative medicine include therapeutic approaches aiming at the protection and/or exogenous administration of both, lung resident and circulating stem/progenitor cells. Local stem/progenitor cells divide to replace injured or postmitotic cells and require strict control in order to regulate their proliferation rate. Traditionally, local endoderm-derived adult stem/progenitor cell population have been considered to reside in well-delineated niches and categorized by lung region (Otto 2002; Liu and Engelhardt 2008; Stripp 2008; Rawlins et al. 2008). Lung cell populations that have been attributed stem/progenitor cell functions

(reviewed by Rawlins and Hogan 2006; Bertoncello and McQualter 2010) include airway epithelial cell types (basal and parabasal cells), Clara cells and type II alveolar epithelial cells (AT2) for the endoderm-derived lineages and side population (SP) cells, pulmonary neuroendocrine cells (PNECs) and endothelial progenitor cells (EPCs) for the homeostasis of mesoderm-derived compartments (vasculature, airway smooth muscle cells, fibroblasts). The models used to investigate the lung stem/progenitor cell populations employ lung injury-inducing agents such as ozone, oxygen, naphthalene, polydocanol, sulphur dioxide, nitrogen dioxide, bleomycin. Both basal cells and secretory cells have displayed progenitor-like behavior following chemical-induced lung injury, and the common feature generally employed to functionally define these cells has been their ability to incorporate [H3]-thymidine into their DNA (reviewed by Crystal et al. 2008; Martin 2008; Snyder et al. 2009). A recent addition to these studies is a report indicating that the human lung may harbor c-kit + resident stem cells capable to regenerate both pulmonary epithelium and vasculature following transplantation into cryoinjured mouse lungs (Kajstura et al. 2011).

Basal and parabasal cells can be found in the proximal airway epithelium, typically in the submucosal glandular ducts and intercartilaginous zone. Studies involving the use of polydocanol or sulphur dioxide (Borthwick et al. 2001) as lung epithelium toxicants have revealed the existence of a cytokeratin 5/14-expressing subpopulation of cells that displayed both clonogenic capacity and multilineage differentiation potential, as they have been shown to differentiate into both ciliated and secretory epithelial cells when cultured at air-liquid interface (Schoch et al. 2004) and repopulate airway epithelium following injury (Hong et al. 2001).

Clara cells are secretory epithelial cells expressing Clara cell secretory protein (CCSP, CC10, uteroglobin, Scgb1a1). There is increasing evidence that the traditional Clara cell population in fact consists of two functionally different subpopulations: the "classic" and the type A (also called new or variant) Clara cells. Type A Clara cell (naphthalene-resistant, CCSP-expressing) are able to survive ozone-induced lung injury and naphthalene-induced lung injury (which causes selective ablation of the "classic" Clara cells) and even to actively proliferate to repopulate injured airway epithelium with both mature, quiescent Clara cells and ciliated epithelial cells. Hence, they have been dubbed facultative transit-amplifying progenitor cells. Although CCSP-expressing cells, they differ from their mature counterparts by lack of secretory granules and smooth endoplasmic reticulum. These cells can also retain labeled DNA precursors (Rawlins et al. 2008). Moreover, there is data indicating that these cells may also co-express SP-C and therefore could represent a common progenitor for Clara cells and AT2 cells. Therefore, they have been termed "bronchioalveolar stem cells" (BASC) and proposed to be strategically located at the bronchioalveolar junction, along with the other localization in the proximity of neuroepithelial bodies (NEBs) (Giangreco et al. 2002). Loss of SP-C expression, simultaneous with the acquirement of aquaporin 5 (a type I alveolar epithelial – AT1 cell marker) by these cells in culture reinforces this hypothesis.

The proposed cell surface phenotype of this cellular subset is Sca1+/CD34+/CD45−/CD31− (Kim et al. 2005; Kim 2007). Another recent candidate multipotent stem / progenitor cell, which displays the EpCAM(hi)/CD104+/CD24(low) phenotype, has also been shown to giverise to bronchial and alveolar epithelium (McQualter et al. 2010).

Type II alveolar epithelial cells (AT2) are the sole source of surfactant and differ both phenotypically and functionally from their type I counterparts. AT2 cells proliferate and generate AT1 cells following injury and have therefore long been considered the putative AT1 progenitors (Adamson and Bowden 1974; Reddy et al. 2004). However, AT1 have also been shown to differentiate into AT2 *in vitro* (Danto et al. 1995), which suggests that AT1 and AT2 may be alternate progenitor cells depending on the type of lung injury. As knowledge and technical tools in stem cell biology improve, a better characterization of the distal lung progenitor cell will be possible, ultimately leading to therapeutic interventions aimed at selective protection of these cells or the use of these cells for cell-based therapies.

Side population (SP) cells have initially been brought into the limelight by their ability to efflux the DNA dye Hoechst (Giangreco et al. 2004). These cells have been identified so far in several organs, including the lung. They seem to be a heterogeneous population initially derived from the bone marrow and display differentiation abilities along both endoderm- and mesoderm-derived lineages (Summer et al. 2004; Martin et al. 2008). In newborn mice, SP cells demonstrate endothelial potential and the number of SP cells decreases in oxygen-induced arrested alveolar growth in these mice, suggesting that changes in lung SP cells may limit their ability to effectively contribute to lung recovery (Irwin et al. 2007). The therapeutic potential of SP cells for lung diseases remains unexplored.

Pulmonary neuroendocrine cells (PNECs) are enigmatic cells, mostly explored for their oxygen-sensing capabilities (Youngson 1993). More recent evidence suggests a role in lung regeneration as well. PNECs proliferate following naphthalene-induced lung injury. PNECs constitutively express calcitonin gene-related peptide (CGRP) and are found along with type A Clara cells in NEBsassociated regenerative foci that appear following chemically-induced lung injury (Reynolds et al. 2000). However, the use of mouse models in which ablation of both naphthalene-sensitive and -resistant Clara cells was possible did not lead to regeneration of airway epithelium, suggesting that these cells are not airway epithelial progenitors (Hong et al. 2001).

Endothelial progenitor cells (EPCs) have been traditionally considered to be circulating cells that contribute to the homeostasis of the endothelium (reviewed by Ingram et al. 2005; Yoder et al. 2007; Yoder and Ingram 2009). Recent exciting findings have identified the presence of resident EPCs within the pulmonary microvascular endothelium with angiogenic capacity (Alvarez et al. 2008), highlighting the potential of new tools in stem cell biology to identify resident lung progenitor cells. The significance of these cells in health and disease as well as their therapeutic potential is currently being explored.

34.5 Therapeutic Potential of Exogenous Stem/Progenitor Cells

34.5.1 Cell Replacement

Beside local stem/progenitor cell populations, there is evidence that non-resident stem/progenitor cells contribute to lung repair following injury (reviewed by Prockop et al. 2003; Neuringer and Randell 2004; Gomperts and Strieter 2007; Weiss et al. 2006; Siniscalco et al. 2008; Warburton et al. 2008; Mora and Rojas 2008; Prockop 2009; Summer and Fine 2008; Sueblinvong and Weiss 2009). Kotton et al. (2001) and Krause et al. (2001) showed that bone marrow-derived stem cells can give rise to "daughter" cells in the airways. This ability of the cells to engraft and differentiate has led to the hypothesis that they may reconstitute injured tissues by replacing the damaged cells. There is now a large body of evidence in support of the hypothesis that bone marrow-derived multipotent stem cells, either hematopoietic (HSCs) or mesenchymal (MSCs), can differentiate into airway (Wang et al. 2005; Wong et al. 2007, 2009) or alveolar epithelial cells *in vitro*, engraft and differentiate *in vivo* and prevent lung injury in various disease models including bleomycin-induced lung fibrosis (Ortiz et al. 2008; Rojas et al. 2005), lipopolysaccharide-induced ALI/ARDS (Yamada et al. 2004, 2005; Gupta et al. 2007; Mei et al. 2007; Xu et al. 2007), oxygen-induced BPD (van Haaften et al. 2009; Aslam et al. 2009), radiation (Abe et al. 2003)- and naphthalene (Serikov et al. 2007)-induced lung injury. This ability of MSCs to differentiate into lung epithelial cells could be harnessed for diseases such as cystic fibrosis, in which the symptoms are caused by mutations in the gene encoding for the cystic fibrosis transmembrane regulator (CFTR), a chloride channel typically expressed in epithelia. The stem cells would be engineered to overexpress functional CFTR and act as a delivery vehicle to the damaged tissues, including the lung (Wong et al. 2007; Bruscia et al. 2009). The same approach would be applicable for other monogenic diseases that severely affect the lung such as alpha-1-antitrypsin deficiency (that leads to irreversible emphysema-like lesions) or surfactant protein B deficiency (resulting in fatal respiratory failure in newborns). Moreover, the possibility of isolating MSCs from other sources, such as the cord blood, makes autologous therapy a very promising approach in treating pediatric patients in the close future.

While most investigators have explored the therapeutic potential of bone marrow-derived MSCs, there is increasing evidence that EPCs may also contribute to the maintenance of the lung parenchyma. This is consistent with observations in experimental models and in the clinic indicating that the number of circulating EPCs correlates with survival and disease severity (Toshner et al. 2009).

Experimental observations suggest that EPCs contribute to lung repair (Fadini et al. 2007). Lipopolysaccharide (LPS)-induced murine lung injury is associated with a rapid release of EPCs into the circulation. These EPCs may collaborate with other bone marrow-derived progenitor cells to promote lung repair (Yamada et al. 2004). In elastase-induced emphysematous lung injury, cells derived from the bone marrow develop characteristics of endothelial cells and contribute to repair the

alveolar capillary wall (Ishizawa et al. 2004a, b; Abe et al. 2004). Likewise, arrested alveolar growth in experimental hyperoxic-induced lung injury in newborn mice, mimicking BPD, is associated with decreased circulating, lung and bone marrow EPC (Balasubramaniam et al. 2007). Interestingly, hyperoxic adult mice did not display alveolar damage and had increased circulating EPCs, implying that decreased EPCs may contribute to the arrested lung growth seen in the neonatal animals.

In patients, the number of circulating EPCs correlates with survival and disease severity in acute lung injury (Burnham et al. 2005), severe COPD or restrictive lung diseases (Fadini et al. 2006), idiopathic pulmonary fibrosis (JunHui et al. 2008) and pneumonia (Yamada et al. 2005).

These observations suggest that EPCs contribute to the repair of injured endothelium and help restore lung integrity and are consistent with previous findings demonstrating the beneficial effect of angiogenic growth factors in experimental BPD (Thébaud et al. 2005; Kunig 2005).

34.5.2 Cell Replacement Versus Paracrine Activity of Stem Cells

However, all the studies aimed at evaluating the therapeutic potential of stem cell transplantation by cell replacement in animal models of lung disease shared one common feature: the degree of stem cell engraftment in the target organs was generally low and therefore alternate mechanisms may account for the observed therapeutic benefit. Moreover, MSCs have been shown effective in inflammatory diseases, such as sepsis (Németh et al. 2009) and asthma (Nemeth et al. 2010), where the local cell engraftment may not be the primary beneficial component. This has led to the current view that stem cells act through a paracrine mechanism by secreted factors (Prockop 2009). Indeed, MSCs secrete anti-apoptotic, angiogenic, and immuno-modulatory factors (Le Blanc et al. 2008; Iyer and Rojas 2008). This paracrine activity has now extensively been explored *in vitro* (Gupta et al. 2007; Hung et al. 2007; Ortiz et al. 2007; Parekkadan et al. 2007; van Haaften et al. 2009) showing cell-protective, pro-angiogenic and anti-inflammatory properties. *Ex vivo* (Lee et al. 2009) and *in vivo* in oxygen- (Aslam et al. 2009) and LPS- (Ionescu et al. 2012b) induced lung injury, MSC-derived conditioned medium conferred the same therapeutic benefit than whole cell therapy. The immunomodulatory, paracrine activity of MSCs may also have therapeutic potential in allergic diseases such as asthma (Ionescu et al. 2012a). Several factors found in the MSCs secretome, among which are interleukin-10 (Németh et al. 2009), transforming growth factor-beta (Nemeth et al. 2010), stanniocalcin-1 (Block et al. 2009), keratinocyte growth factor (Lee et al. 2009) and adiponectin (Ionescu et al. 2012a), have been proposed to mediate MSCs cross-talk with various effector cell types. Identification of these secreted factors, along with clarification of their mechanisms of action, may allow the development of new treatments.

34.5.3 ES and Induced Pluripotent Stem (iPS) Cells

ES cells represent the most pluripotent stem cells but are mired in controversy. The recent landmark generation of "ES-like", induced pluripotent stem cells (iPS) using viral delivery of pluripotency genes to somatic cells (Takahashi and Yamanaka 2006) may relieve many ethical concerns related to the use of ES cells for research and has opened the way to large-scale production and evaluation of pluripotent stem cells for lung regeneration and repair.

When maintained in conditions that support the undifferentiated state, pluripotent stem cells show unlimited proliferation potential, which renders them ideal candidates for studies in developmental biology regeneration (Varanou et al. 2008). ES cells can be directed to differentiate into definitive endoderm from which they may be further differentiated into lung cells using specific factors (Rippon et al. 2006). Another method employed was the exposure of ES cells to microenvironments mimicking lung conditions (coculture with lung mesenchyme or lung cell extracts) (Van Vranken et al. 2005). Although there are isolated reports indicating the attainment of fully differentiated proximal airway-like tissue (Coraux et al. 2005), airway epithelium (Samadikuchaksaraei and Bishop 2006) or even pure populations of AT2 cells (Wang et al. 2007a, b) from ES cells, most of the available literature indicates cellular heterogeneity of the cultures with a relatively low yield of lung cell. *In vivo* administration of ES or progenitor cells derived from ES or iPS cells has also generated inconclusive results so far, with limited and transient ES cell expression in the lung (reviewed by Rippon et al. 2008; Wetsel et al. 2011). Further steps, such as stable differentiation and purification of desired cell populations need to be taken in order to assess the potential of ES and iPS cells for lung diseases.

34.5.4 Stem Cells and Carcinogenesis

The term "lung cancer" encompasses several different pathological entities: squamous cell carcinomas, small cell carcinomas and adenocarcinomas which appear with different frequency in different areas of the lung, suggesting that local lung environment may act upon cell fate. The hypothesis of tumor-initiating cells (Cancer stem cells) could explain the relapse of certain tumors owing to the fact that these cells might be resistant to many conventional cancer therapies (Peacock and Watkins 2008). The identification of putative resident stem cells in lung tumors (Kim et al. 2005) leads to the question whether the resident cells that survive pollutant-induced injury may in fact be such a cancer stem cells. The existence of cancer stem cells in the lung is supported by work indicating that CD133+ is a marker of self-renewing cells that sustain tumor propagation in mice (Eramo et al. 2007). Although the proportion of cells expressing this marker lacks prognostic value (Salnikov et al. 2009), these cells are resistant to cisplatin treatment (Bertolini et al. 2009). Other work suggests that activation of the k-ras gene, whose mutation is considered to be directly linked to lung cancer in humans (Johnson et al. 2001), upregulates the

SP-C+/CCSP+(BASC) cells and leads to development of lung adenocarcinomas (Kim et al. 2005). Similarly, deletion of PTEN, PI3 kinase or p38a MAP kinase led to proliferation of SP-C+/CCSP+cells simultaneous with the increase in susceptibility to develop lung neoplasms (Yanagi et al. 2007), whereas Bmi1 deletion had opposite effects (Dovey et al. 2008). However, it has not yet been clearly determined whether there is a link between the CD133-expressing and the dual SP-C/CCSP-expressing cell population or whether either of these populations acts as an initiator or propagator of lung malignant tumors. Also, the cells in small cell carcinomas have been shown to express basal cell markers, whereas small cell carcinomas have been found to express markers reminiscent of PNECs (Giangreco et al. 2007), but the direct relationship between the putative stem/progenitor cells and the neoplastic cells has yet to be investigated. Although much work is still needed to identify and characterize cancer stem cells-initiating cells, the discovery opens therapeutic avenues for designing specific cellular targets for the treatment of cancer (Pine et al. 2008; Yagui-Beltrán et al. 2008; Alison et al. 2009; Gao and Mittal 2009; Zhou et al. 2009).

34.6 Biotechnology – Engineering Lung Tissue

Currently, lung transplantation is the only viable solution for incurable lung disease in patients under 65 years of age. These lung diseases include lung fibrosis COPD, CF, primary pulmonary hypertension, sarcoidosis, lymphangioleiomyomatosis. However, the mortality rate from the moment the potential recipients are placed on the waiting list until they receive the transplant is currently around 30 % (Punch et al. 2007). Moreover, lung transplantation is not an option for patients with other major accompanying health problems. This highlights the necessity to seek for alternative approaches, such as the development of the artificial lung or bioengineered lung components.

34.6.1 Human Ex-Vivo Lung Project (HELP)

Currently, the supply of donor lungs does not match the demand and one of the facts that contribute to this shortage is that only about 20 % of donor organs are considered acceptable for transplantation (Punch et al. 2007). Improper oxygenation capacity (reflected by a PaO_2 below 300 mmHg after oxygenation with a FiO_2 of 100 % for 5 min and PEEP greater than 5 cm H_2O) leads to rejection of donor lungs. HELP involves the concept of reconditioning and transplantation of these otherwise rejected donor lungs. Lungs are reconditioned *ex vivo* by continuous perfusion with a lung evaluation–preservation solution (Steen solution – Wierup et al. 2006) mixed with erythrocytes for several hours, until the functional parameters reach acceptable values. After reconditioning, these lungs can be transplanted immediately or stored at

8 °C in *ex vivo* extracorporeal membrane oxygenation (ECMO) until transplantation can be performed (Ingemansson et al. 2009). The first transplant of lungs harvested from a donor and reconditioned *ex vivo* was performed successfully in 2007 (Steen et al. 2007). The impact of this promising strategy remains to be evaluated.

34.6.2 Artificial Lung – NovaLung ®

The artificial lung is a relatively new method, similar in concept to dialysis and designed to support respiratory function while the potential lung transplant recipient is waiting for the donor lungs (Fischer et al. 2006). The patient's blood flows into a device that removes carbon dioxide and enriches the blood in oxygen. As compared to conventional ECMO, the artificial lung eliminates the need for an extracorporeal blood pump and can be used for extended periods of time (up to 100 days – von Mach et al. 2006) in centres where ECMO is not available. Other advantages of this system over ECMO are reduced anticoagulation and avoidance of long-term mechanical ventilation (Taylor and Holtby 2009; review by Walles 2007).

34.6.3 Bioengineered Lung Tissue

The structural and functional complexity of the lung has so far restricted the development of bioengineered lung tissue, when compared to the progress made in engineering less complex organs, such as the skin or the urinary bladder (Atala 2007). A recent *in silico* model of the alveolar-capillary interface has been developed employing biomaterials and human alveolar epithelial cells at air-liquid interface, along with human pulmonary microvascular endothelial cells (Huh et al. 2010). This type of biomimetic microsystems could facilitate drug screening and toxicology studies by allowing high-throughput processing. On a larger scale, so far both ES cells and adult multipotent stem cells, as well as mixed cell populations containing progenitor cells or terminally differentiated cells such as fibroblasts or chondrocytes have been used with promising results to generate lung cell lineages or bioengineered lung components (Barrilleaux et al. 2006). Moreover, the ability of MSCs to differentiate along the chondrogenic lineage has so far been harnessed to engineer a main bronchus using the tracheal acellular scaffold (Macchiarini et al. 2008). The engineered bronchus was successfully transplanted into a patient whose own airway had been irreversibly damaged. However, the lack of conclusive information with respect to the tumorigenic potential of stem cells, especially ES cells, known for their karyotypic instability, together with the unanswered question regarding the local progenitor cells as potential cancer stem cells, demand careful safety evaluation of stem cell-based approaches. Also, the biomaterials used as scaffolds on which the lung tissue would be grown need to be evaluated with regards to their biocompatibility in terms

of elasticity, adsorption kinetics, porosity and degradation kinetics (Nichols and Cortiella 2008). So far, natural scaffolds of type I collagen, Matrigel (composed of basement membrane proteins), Gelfoam (derived from porcine skin gelatin) and synthetic polymers, such as polyglycolic acid (PGA) have been used in attempts to engineer lung tissue. Aside from constructed scaffolds, a recent breakthrough in lung bioengineering has been achieved by demonstrating that decellularized lung matrices have the ability to support repopulation with newly-seeded epithelial and endothelial cells and, moreover, to sustain lung function following transplantation into animals (Ott et al. 2010; Petersen et al. 2010; Song et al. 2011).

34.7 Clinical Studies: Experience, Outcome, Limitations

Several limitations have hampered clinical trials of stem cell based therapies for lung diseases. There are certain risks to heterologous cell transplantation. The cells may carry infectious agents, which poses an even enhanced peril in the case of recipients who have developed graft-versus-host disease (Runde et al. 2001). Furthermore, there have been reports of bronchiolitis obliterans organizing pneumonia (BOOP) in patients who had undergone HSC transplantation (Hildebrandt et al. 2008). Both heterologous and autologous transplantation bear the risk of tumor formation. ES and iPS cells develop teratomas *in vivo* and there are also reports indicating that transplantation of neural stem cells led to the development of tumors in the recipient brain (Amariglio et al. 2009). MSCs, generally considered less prone to acquiring karyotypic abnormalities compared to ES cells, may also pose tumorigenic risks (Aguilar et al. 2007; Tolar et al. 2007). However, these dangers may be overcome: recent findings indicating that stem cell-secreted factors exert therapeutic benefits may abrogate the need to deliver the cells themselves to the damaged tissues.

Another limitation is the insufficient characterization of stem cells in terms of both phenotype and function. For MSCs, minimal criteria for defining human MSCs, established by the International Society for Cellular Therapy (Dominici et al. 2006), have reduced some of the variations with regards to cellular composition of MSC populations isolated according to different protocols. Lung injury prevention obtained with MSCs in various animal models of lung disease, together with their ease of isolation and culture, as well as their immuno-modulatory properties make these cells very promising candidates for clinical trials.

Thus far, stem cells have been transplanted in humans as part of whole bone marrow transplantation for various disorders (including leukemia and genetic diseases of the immune system). Gender-mismatched transplantation (male donor bone marrow to female recipient) has proven to be a useful tool in assessing the impact of stem cell transplantation on other organs than bone marrow. Donor male cells were identified in the lungs of recipients as epithelial and endothelial cells (Suratt et al. 2003) and also in the liver (Theise et al. 2000), heart (Deb et al. 2003), brain (Mezey et al. 2003; Crain et al. 2005) and kidney (Poulsom et al. 2001). Also, in the

reverse case where males were recipients of sex-mismatched organ transplants, the Y chromosome indicating recipient origin was identified in a variable proportion of organ-specific cells. With regards to lungs, the chimerism was present in bronchial epithelial cells, AT2 and seromucous glands (Prockop et al. 2003).

Currently, one phase I clinical trial aimed at evaluating the tolerability and safety of progenitor cells for the treatment of pulmonary arterial hypertension (Pulmonary Hypertension: Assessment of Cell Therapy, PHACeT) is underway. Autologous endothelial progenitor cells are engineered *ex vivo* to express endothelial nitric oxide synthase (eNOS), followed by injection of the cells via a pulmonary artery line. Previous pilot studies have supported the feasibility of this approach in idiopathic pulmonary hypertension (Wang et al. 2007a, b; Zhu et al. 2008).

On the basis of initial reports of safety and efficacy following allogeneic administration of MSCs to patients with Crohn's disease or with graft-versus-host disease, a trial studying the effect of MSCs in patients with COPD is ongoing. Further information on current clinical trials involving the use of stem cells or stem cell-derived products are regularly updated on the United States National Institute of Health's website www.ClinicalTrials.gov.

34.8 Conclusions and Future Perspectives on Lung Regenerative Therapies

Since the initial observations that stem cells engraft into the lung (Kotton et al. 2001; Krause et al. 2001), stem cell based therapies (using mostly whole bone marrow derived cells or MSCs) have been studied extensively in various animal models of lung diseases. All of them showed the ability of these cells to prevent lung injury, despite a low rate of cell engraftment. The current hypothesis is that stem cells activate a paracrine mechanism protecting resident lung cells from injury, rather than through engraftment and cell replacement. The identification of soluble factors produced by MSCs may yield new therapeutic avenues for lung diseases that currently lack efficient treatment strategies, thereby alleviating the potential risks associated with whole-cell delivery.

Stem cells may also hold promise for genetic diseases such as cystic fibrosis and alpha-1-antitrypsin deficiency. Stem cells engineered to express the corrected genes could be differentiated *in vitro* or *in vivo* and confer sufficient gene function. The projected developments of iPS cell research will facilitate further investigation of cell-based approaches for therapeutic purposes but also for understanding of diseases processes and drug testing. The challenge of lung regeneration, e.g. repair of established lung damage, relevant for lung fibrosis and emphysema for example, remains and may require additional strategies in combination with stem cell-based approaches to "rebuild" the lung. It is hoped that insight into lung stem cell biology will facilitate and expand bioengineering approaches for lung regeneration. Recommendations for future directions for lung stem cell biology have been summarized by Weiss and colleagues (Weiss et al. 2008). While much more needs to be

learned about the mechanisms of normal lung development-injury-and-repair, stem cell biology and the long term efficacy and safety of stem cell-based therapies, the promising animal and sparse clinical observations suggest that it might be time to initiate clinical trials using stem cell-based approaches for devastating lung diseases that currently lack effective therapies. There is ample precedent in medicine of established treatments for which the mechanism of action is still not fully understood. Carefully conducted trials for patients in desperate need for improvement may teach us more than additional pre-clinical studies.

References

Abe S et al (2003) Transplanted BM and BM side population cells contribute progeny to the lung and liver in irradiated mice. Cytotherapy 5:523–533

Abe S et al (2004) Cells derived from the circulation contribute to the repair of lung injury. Am J Respir Crit Care Med 170:1158–1163

Adamson IY, Bowden DH (1974) The type 2 cell as progenitor of alveolar epithelial regeneration. A cytodynamic study in mice after exposure to oxygen. Lab Invest 30:35–42

Aguilar S et al (2007) Murine but not human mesenchymal stem cells generate osteosarcoma-like lesions in the lung. Stem Cells 25(6):1586–1594

Alison MR, Lebrenne AC, Islam S (2009) Stem cells and lung cancer: future therapeutic targets? Expert Opin Biol Ther 9:1127–1141

Alvarez DF et al (2008) Lung microvascular endothelium is enriched with progenitor cells that exhibit vasculogenic capacity. Am J Physiol Lung Cell Mol Physiol 294(3):L419–L430

Amariglio N et al (2009) Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med 6(2):e1000029

Aslam M et al (2009) Bone marrow stromal cells attenuate lung injury in a murine model of neonatal chronic lung disease. Am J Respir Crit Care Med 180(11):1122–1130. doi:10.1164/rccm.200902-0242OC

Atala A (2007) Engineering tissues, organs and cells. J Tissue Eng Regen Med 1(2):83–96

Balasubramaniam V et al (2007) Hyperoxia reduces bone marrow, circulating, and lung endothelial progenitor cells in the developing lung: implications for the pathogenesis of bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol 292:L1073–L1084

Barrilleaux B et al (2006) Review: ex vivo engineering of living tissues with adult stem cells. Tissue Eng 12(11):3007–3019

Bertolini G et al (2009) Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. Proc Natl Acad Sci USA 106:16281–16286

Bertoncello I, McQualter J (2010) Endogenous lung stem cells: what is their potential for use in regenerative medicine? Expert Rev Respir Med 4(3):349–362

Block GJ et al (2009) Multipotent stromal cells are activated to reduce apoptosis in part by upregulation and secretion of stanniocalcin-1. Stem Cells 27(3):670–681

Borthwick DW et al (2001) Evidence for stem-cell niches in the tracheal epithelium. Am J Respir Cell Mol Biol 24(6):662–670

Bruscia EM et al (2009) Macrophages directly contribute to the exaggerated inflammatory response in cystic fibrosis transmembrane conductance regulator−/− mice. Am J Respir Cell Mol Biol 40(3):295–304

Burnham EL et al (2005) Increased circulating endothelial progenitor cells are associated with survival in acute lung injury. Am J Respir Crit Care Med 172(7):854–860

Burri PH (2006) Structural aspects of postnatal lung development – alveolar formation and growth. Biol Neonate 89(4):313–322

Cardoso WV, Whitsett JA (2008) Resident cellular components of the lung: developmental aspects. Proc Am Thorac Soc 5:767–771
Coraux C et al (2005) Embryonic stem cells generate airway epithelial tissue. Am J Respir Cell Mol Biol 32(2):87–92
Crain BJ, Tran SD, Mezey E (2005) Transplanted human bone marrow cells generate new brain cells. J Neurol Sci 233(1–2):121–123
Crystal RG et al (2008) Airway epithelial cells: current concepts and challenges. Proc Am Thorac Soc 5:772–777
Danto SI et al (1995) Reversible transdifferentiation of alveolar epithelial cells. Am J Respir Cell Mol Biol 12(5):497–502
Deb A et al (2003) Bone marrow-derived cardiomyocytes are present in adult human heart: a study of gender-mismatched bone marrow transplantation patients. Circulation 107(9):1247–1249
Dominici M et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8(4):315–317
Dovey JS et al (2008) Bmi1 is critical for lung tumorigenesis and bronchioalveolar stem cell expansion. Proc Natl Acad Sci USA 105:11857–11862
Eramo A et al (2007) Identification and expansion of the tumorigenic lung cancer stem cell population. Cell Death Differ 15:504–514
Fadini GP et al (2006) Circulating progenitor cells are reduced in patients with severe lung disease. Stem Cells 24(7):1806–1813
Fadini GP et al (2007) The emerging role of endothelial progenitor cells in pulmonary hypertension and diffuse lung diseases. Sarcoidosis Vasc Diffuse Lung Dis 24:85–93
Fischer S et al (2006) Bridge to lung transplantation with the novel pumpless interventional lung assist device NovaLung. J Thorac Cardiovasc Surg 131(3):719–723
Gao D, Mittal V (2009) The role of bone-marrow-derived cells in tumor growth, metastasis initiation and progression. Trends Mol Med 15:333–343
Giangreco A, Reynolds SD, Stripp BR (2002) Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. Am J Pathol 161:173–182
Giangreco A et al (2004) Molecular phenotype of airway side population cells. Am J Physiol Lung Cell Mol Physiol 286(4):L624–L630
Giangreco A, Groot KR, Janes SM (2007) Lung cancer and lung stem cells: strange bedfellows? Am J Respir Crit Care Med 175:547–553
Gomperts BN, Strieter RM (2007) Stem cells and chronic lung disease. Annu Rev Med 58:285–298
Gupta N et al (2007) Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. J Immunol 179:1855–1863
Hildebrandt GC et al (2008) Recipient NOD2/CARD15 variants: a novel independent risk factor for the development of bronchiolitis obliterans after allogeneic stem cell transplantation. Biol Blood Marrow Transplant 14(1):67–74
Hong KU et al (2001) Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. Am J Respir Cell Mol Biol 24(6):671–681
Huh D et al (2010) Reconstituting organ-level lung functions on a chip. Science 328(5986):1662–1668
Hung SC et al (2007) Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. Stem Cells 25(9):2363–2370
Ingemansson R et al (2009) Clinical transplantation of initially rejected donor lungs after reconditioning ex vivo. Ann Thorac Surg 87(1):255–260
Ingram DA, Caplice NM, Yoder MC (2005) Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. Blood 106(5):1525–1531
Ionescu LI et al (2012a) Airway delivery of soluble factors from plastic-adherent bone marrow cells prevents murine asthma. Am J Respir Cell Mol Biol 46(2):207–216

Ionescu L et al (2012b) Stem cell conditioned medium improves acute lung injury in mice: in vivo evidence for stem cell paracrine action. Am J Physiol Lung Cell Mol Physiol 303(11):L967–977
Irwin D et al (2007) Neonatal lung side population cells demonstrate endothelial potential and are altered in response to hyperoxia-induced lung simplification. Am J Physiol Lung Cell Mol Physiol 293(4):L941–L951
Ishizawa K, Kubo H, Yamada M et al (2004a) Bone marrow-derived cells contribute to lung regeneration after elastase-induced pulmonary emphysema. FEBS Lett 556:249–252
Ishizawa K, Kubo H, Yamada M et al (2004b) Hepatocyte growth factor induces angiogenesis in injured lungs through mobilizing endothelial progenitor cells. Biochem Biophys Res Commun 324:276–280
Iyer SS, Rojas M (2008) Anti-inflammatory effects of mesenchymal stem cells: novel concept for future therapies. Expert Opin Biol Ther 8:569–581
Johnson L et al (2001) Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. Nature 410:1111–1116
JunHui Z et al (2008) Reduced number and activity of circulating endothelial progenitor cells in patients with idiopathic pulmonary arterial hypertension. Respir Med 102:1073–1079
Kajstura J et al (2011) Evidence for human lung stem cells. N Engl J Med 364(19):1795–1806
Kim CF (2007) Paving the road for lung stem cell biology: bronchioalveolar stem cells and other putative distal lung stem cells. Am J Physiol Lung Cell Mol Physiol 293:L1092–L1098
Kim CFB et al (2005) Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 121:823–835
Kitaoka H, Burri PH, Weibel ER (1996) Development of the human fetal airway tree: analysis of the numerical density of airway endtips. Anat Rec 244:207–213
Kotton DN et al (2001) Bone marrow-derived cells as progenitors of lung alveolar epithelium. Development 128:5181–5188
Krause DS et al (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105:369–377
Kunig AM (2005) Recombinant human VEGF treatment enhances alveolarization after hyperoxic lung injury in neonatal rats. Am J Physiol Lung Cell Mol Physiol 289:L529–L535
Lane S, Rippon HJ, Bishop AE (2007) Stem cells in lung repair and regeneration. Regen Med 2(4):407–415
Le Blanc K et al (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 371:1579–1586
Lee JW et al (2009) Allogeneic human mesenchymal stem cells for treatment of E. coli endotoxin-induced acute lung injury in the ex vivo perfused human lung. Proc Natl Acad Sci USA 106:16357–16362
Liu X, Engelhardt JF (2008) The glandular stem/progenitor cell niche in airway development and repair. Proc Am Thorac Soc 5:682–688
Macchiarini P et al (2008) Clinical transplantation of a tissue-engineered airway. Lancet 372:2023–2030
Maeda Y, Davé V, Whitsett JA (2007) Transcriptional control of lung morphogenesis. Physiol Rev 87:219–244
Martin U (2008) Methods for studying stem cells: adult stem cells for lung repair. Methods 45:121–132
Martin J et al (2008) Adult lung side population cells have mesenchymal stem cell potential. Cytotherapy 10(2):140–151
McQualter JL et al (2010) Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. Proc Natl Acad Sci USA 107(4):1414–1419
Mei SH et al (2007) Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. PLoS Med 4(9):e269
Mezey E et al (2003) Transplanted bone marrow generates new neurons in human brains. Proc Natl Acad Sci USA 100(3):1364–1369
Mora AL, Rojas M (2008) Aging and lung injury repair: a role for bone marrow derived mesenchymal stem cells. J Cell Biochem 105:641–647

Németh K et al (2009) Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med 15(1):42–49
Nemeth K et al (2010) Bone marrow stromal cells use TGF-beta to suppress allergic responses in a mouse model of ragweed-induced asthma. Proc Natl Acad Sci USA 107(12):5652–5657
Neuringer IP, Randell SH (2004) Stem cells and repair of lung injuries. Respir Res 5:6
Nichols JE, Cortiella J (2008) Engineering of a complex organ: progress toward development of a tissue-engineered lung. Proc Am Thorac Soc 5:723–730
Ortiz LA et al (2007) Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. Proc Natl Acad Sci USA 104(26):11002–11007
Ortiz LA et al (2008) Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc Natl Acad Sci USA 100:8407–8411
Ott HC et al (2010) Regeneration and orthotopic transplantation of a bioartificial lung. Nat Med 16(8):927–933
Otto WR (2002) Lung epithelial stem cells. J Pathol 197(4):527–535
Parekkadan B, van Poll D, Suganuma K et al (2007) Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. PLoS One 2(9):e941
Peacock CD, Watkins DN (2008) Cancer stem cells and the ontogeny of lung cancer. J Clin Oncol 26(17):2883–2889
Petersen TH et al (2010) Tissue-engineered lungs for in vivo implantation. Science 329(5991):538–541
Pine SR, Marshall B, Varticovski L (2008) Lung cancer stem cells. Dis Markers 24:257–266
Poulsom R et al (2001) Bone marrow contributes to renal parenchymal turnover and regeneration. J Pathol 195(2):229–235
Prockop DJ (2009) Repair of tissues by adult stem/progenitor cells (MSCs): controversies, myths, and changing paradigms. Mol Ther 17:939–946
Prockop DJ, Gregory CA, Spees JL (2003) One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. Proc Natl Acad Sci USA 100(Suppl 1):11917–11923
Punch JD et al (2007) Organ donation and utilization in the United States, 1996–2005. Am J Transplant 7(5 Pt 2):1327–1338
Rawlins EL (2008) Lung epithelial progenitor cells: lessons from development. Proc Am Thorac Soc 5:675–681
Rawlins EL, Hogan BL (2006) Epithelial stem cells of the lung: privileged few or opportunities for many? Development 133(13):2455–2465
Rawlins EL et al (2008) Epithelial stem/progenitor cells in lung postnatal growth, maintenance, and repair. Cold Spring Harb Symp Quant Biol 73:291–295
Reddy R et al (2004) Isolation of a putative progenitor subpopulation of alveolar epithelial type 2 cells. Am J Physiol Lung Cell Mol Physiol 286:L658–L667
Reynolds SD et al (2000) Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. Am J Pathol 156:269–278
Rippon HJ et al (2006) Derivation of distal lung epithelial progenitors from murine embryonic stem cells using a novel three-step differentiation protocol. Stem Cells 24:1389–1398
Rippon HJ, Lane S, Qin M et al (2008) Embryonic stem cells as a source of pulmonary epithelium in vitro and in vivo. Proc Am Thorac Soc 5(6):717–722
Rojas M et al (2005) Bone marrow-derived mesenchymal stem cells in the repair of the injured lung. Am J Respir Cell Mol Biol 33(2):145–152
Runde V et al (2001) Adenoviral infection after allogeneic stem cell transplantation (SCT): report on 130 patients from a single SCT unit involved in a prospective multi center surveillance study. Bone Marrow Transplant 28(1):51–57
Salnikov AV et al (2009) CD133 is indicative for a resistance phenotype but does not represent a prognostic marker for survival of non-small cell lung cancer patients. Int J Cancer. doi:10.1002/ijc.24822

Samadikuchaksaraei A, Bishop AE (2006) Derivation and characterization of alveolar epithelial cells from murine embryonic stem cells in vitro. Methods Mol Biol 330:233–248
Schoch KG et al (2004) A subset of mouse tracheal epithelial basal cells generates large colonies in vitro. Am J Physiol Lung Cell Mol Physiol 286(4):L631–L642
Serikov VB et al (2007) Evidence of temporary airway epithelial repopulation and rare clonal formation by BM-derived cells following naphthalene injury in mice. Anat Rec (Hoboken) 290:1033–1045
Siniscalco D et al (2008) Stem cell therapy: the great promise in lung disease. Ther Adv Respir Dis 2:173–177
Snyder JC, Teisanu RM, Stripp BR (2009) Endogenous lung stem cells and contribution to disease. J Pathol 217:254–264
Song JJ et al (2011) Enhanced in vivo function of bioartificial lungs in rats. Ann Thorac Surg 92(3):998–1006
Steen S et al (2007) First human transplantation of a nonacceptable donor lung after reconditioning ex vivo. Ann Thorac Surg 83:2191–2194
Stripp BR (2008) Hierarchical organization of lung progenitor cells: is there an adult lung tissue stem cell? Proc Am Thorac Soc 5:695–698
Sueblinvong V, Weiss DJ (2009) Cell therapy approaches for lung diseases: current status. Curr Opin Pharmacol 9:268–273
Summer R, Fine A (2008) Mesenchymal progenitor cell research: limitations and recommendations. Proc Am Thorac Soc 5:707–710
Summer R et al (2004) Translational physiology: origin and phenotype of lung side population cells. Am J Physiol Lung Cell Mol Physiol 287:L477–L483
Suratt BT et al (2003) Human pulmonary chimerism after hematopoietic stem cell transplantation. Am J Respir Crit Care Med 168(3):318–322
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
Taylor K, Holtby H (2009) Emergency interventional lung assist for pulmonary hypertension. Anesth Analg 109(2):382–385
Thébaud B et al (2005) Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. Circulation 112:2477–2486
Theise ND et al (2000) Liver from bone marrow in humans. Hepatology 32(1):11–16
Tolar J et al (2007) Sarcoma derived from cultured mesenchymal stem cells. Stem Cells 25(2):371–379
Toshner M et al (2009) Evidence of dysfunction of endothelial progenitors in pulmonary arterial hypertension. Am J Respir Crit Care Med 180:780–787
van Haaften T et al (2009) Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. Am J Respir Crit Care Med 180(11):1131–1142. doi:10.1164/rccm.200902-0179OC
Van Vranken BE et al (2005) Coculture of embryonic stem cells with pulmonary mesenchyme: a microenvironment that promotes differentiation of pulmonary epithelium. Tissue Eng 11(7–8):1177–1187
Varanou A, Page CP, Minger SL (2008) Human embryonic stem cells and lung regeneration. Br J Pharmacol 155:316–325
von Mach MA et al (2006) An update on interventional lung assist devices and their role in acute respiratory distress syndrome. Lung 184(3):169–175
Walles T (2007) Clinical experience with the iLA Membrane Ventilator pumpless extracorporeal lung-assist device. Expert Rev Med Devices 4(3):297–305
Wang G et al (2005) Adult stem cells from bone marrow stroma differentiate into airway epithelial cells: potential therapy for cystic fibrosis. Proc Natl Acad Sci USA 102(1):186–191
Wang D et al (2007a) A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. Proc Natl Acad Sci USA 104(11):4449–4454

Wang XX et al (2007b) Transplantation of autologous endothelial progenitor cells may be beneficial in patients with idiopathic pulmonary arterial hypertension: a pilot randomized controlled trial. J Am Coll Cardiol 49(14):1566–1571
Warburton D et al (2001) Do lung remodeling, repair, and regeneration recapitulate respiratory ontogeny? Am J Respir Crit Care Med 164(10 Pt 2):S59–S62
Warburton D et al (2008) Stem/progenitor cells in lung development, injury repair, and regeneration. Proc Am Thorac Soc 5:703–706
Weiss DJ et al (2006) Adult stem cells, lung biology, and lung disease. NHLBI/Cystic Fibrosis Foundation workshop. Proc Am Thorac Soc 3:193–207
Weiss DJ et al (2008) Stem cells and cell therapies in lung biology and lung diseases. Proc Am Thorac Soc 5:637–667
Wetsel RA, Wang D, Calame DG (2011) Therapeutic potential of lung epithelial progenitor cells derived from embryonic and induced pluripotent stem cells. Annu Rev Med 62:95–105
Wierup P et al (2006) Ex vivo evaluation of nonacceptable donor lungs. Ann Thorac Surg 81(2):460–466
Wong AP et al (2007) Targeted cell replacement with bone marrow cells for airway epithelial regeneration. Am J Physiol Lung Cell Mol Physiol 293:L740–L752
Wong PM et al (2008) Emphysema in young adult survivors of moderate-to-severe bronchopulmonary dysplasia. Eur Respir J 32(2):321–328
Wong AP et al (2009) Identification of a bone marrow-derived epithelial-like population capable of repopulating injured mouse airway epithelium. J Clin Invest 119:336–348
World Health Organization (WHO) World Health Report (2000)
Xu J et al (2007) Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice. Am J Physiol Lung Cell Mol Physiol 293(1):L131–L141
Yagui-Beltrán A, He B, Jablons DM (2008) The role of cancer stem cells in neoplasia of the lung: past, present and future. Clin Transl Oncol 10:719–725
Yamada M et al (2004) Bone marrow-derived progenitor cells are important for lung repair after lipopolysaccharide-induced lung injury. J Immunol 172:1266–1272
Yamada M et al (2005) Increased circulating endothelial progenitor cells in patients with bacterial pneumonia: evidence that bone marrow derived cells contribute to lung repair. Thorax 60:410–413
Yanagi S et al (2007) Pten controls lung morphogenesis, bronchioalveolar stem cells, and onset of lung adenocarcinomas in mice. J Clin Invest 117:2929–2940
Yoder MC, Ingram DA (2009) Endothelial progenitor cell: ongoing controversy for defining these cells and their role in neoangiogenesis in the murine system. Curr Opin Hematol 16(4):269–273
Yoder MC et al (2007) Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 109(5):1801–1809
Youngson C (1993) Oxygen sensing in airway chemoreceptors. Nature 365(6442):153–155
Zeltner TB, Burri PH (1987) The postnatal development and growth of the human lung. II. Morphology. Respir Physiol 67:269–282
Zhou BB et al (2009) Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. Nat Rev Drug Discov 8(10):806–823
Zhu JH et al (2008) Safety and efficacy of autologous endothelial progenitor cells transplantation in children with idiopathic pulmonary arterial hypertension: open-label pilot study. Pediatr Transplant 12(6):650–655

Chapter 35
Vascular Regeneration: Endothelial Progenitor Cell Therapy for Ischemic Diseases

Masaaki Ii, Atsuhiko Kawamoto, and Takayuki Asahara

Abstract Since the discovery of circulating endothelial progenitor cells (EPC) in adult human peripheral blood, EPCs are believed to home to sites of neovascularization, where they contribute to vascular regeneration by forming a structural component of capillaries and by secreting angiogenic factors, thereby enhancing vascular and blood flow recovery in ischemic tissue. This therapeutic strategy has been effective in animal models of ischemia, and we and other clinical trials have demonstrated that it was safe and feasible for treatment of critical ischemic limb and cardiovascular diseases. However, the decline of EPCs in the peripheral blood and evidence that several disease states reduced EPC number and/or function have prompted the development of several strategies to overcome these limitations, including the administration of genetically modified EPCs that overexpress angiogenic growth factors. To optimize therapeutic outcomes, investigators must keep refining methods of EPC purification, expansion, and administration, and to develop techniques that overcome the intrinsic decline and phenotypic deficiencies of EPCs. In this chapter, we have illustrated EPC biology and the therapeutic potential of EPCs for vascular regeneration demonstrating our data of clinical study.

M. Ii, M.D., Ph.D.
Department of Pharmacology, Group of Translational Stem Cell Research, Osaka Medical College, 2-7, Daigaku-machi, Takatsuki, Osaka 569-8686, Japan

A. Kawamoto, M.D., Ph.D.
Unit of Regenerative Medicine, Institute of Biomedical Research and Innovation/RIKEN Center for Developmental Biology, 2-2, Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047, Japan

T. Asahara, M.D., Ph.D. (✉)
Department of Regenerative Medicine Science, Tokai University School of Medicine, 143, Shimokasuya, Isehara, Kanagawa 259-1193, Japan

Group of Vascular Regeneration Research, Institute for Biomedical Research and Innovation (IBRI)/RIKEN Center for Developmental Biology, 2-2 Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047, Japan
e-mail: asa777@is.icc.u-tokai.ac.jp

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_35,

35.1 Introduction

Recently, endothelial progenitor cells (EPCs) have been isolated from adult human peripheral blood (PB) (Asahara et al. 1997). EPCs are shown to be derived from bone marrow (BM), and to accumulate in active angiogenic foci and participate in neovascularization following ischemic insults, (Asahara et al. 1999a, b) exhibiting common stem/progenitor cell characteristics. The evidence that BM derived EPCs home to sites of neovascularization differentiating into endothelial cells (ECs) *in situ* is consistent with "vasculogenesis", a critical paradigm well described in embryonic neovascularization, but recently proposed in adults in which a reservoir of stem/progenitor cells contribute to postnatal vascular formation. The discovery of EPCs has therefore drastically changed our understanding of adult blood vessel formation specifically in ischemic tissue. The following issue highlights the potential utility of EPCs for therapeutic angio/vasculogenesis in ischemic diseases, updating the notion of EPC biology.

35.1.1 Post-natal Neovascularization

In the events of minor scale neovascularization such as slight wounds or burns, "*in situ* preexisting ECs" causing post-natal angiogenesis may replicate and replace the existing cell population enough, as ECs exhibit the ability for self-repair that preserves their proliferative activity. Neovascularization through differentiated ECs, however, is limited in terms of cellular life span (Hayflick limit) and their inability to incorporate into remote target sites. In the case of large scale tissue repair, such as the patients who experienced acute vascular insult secondary to burns, coronary artery bypass grafting (CABG), or acute myocardial infarction, (Gill et al. 2001; Shintani et al. 2001) or in physiological cyclic organogenesis of endometrium, (Asahara et al. 1999a) BM- derived or *in situ* EPC kinetics are activated under the influence of appropriate cytokines, hormones and/or growth factors through the autocrine, paracrine, and/or endocrine systems. Thus the contemporary view of tissue regeneration is that neighboring differentiated ECs are relied upon for vascular regeneration during a minor insult, whereas tissue specific or BM-derived stem/progenitor cells bearing EPCs/ECs are crucial when an emergent and larger scale vascular regeneration process is required (Fig. 35.1).

35.1.2 Biological Characteristics of EPCs

During embryonic development, blood islands initially fuse to form a yolk sac capillary network, (Risau and Flamme 1995) which provides the foundation for an

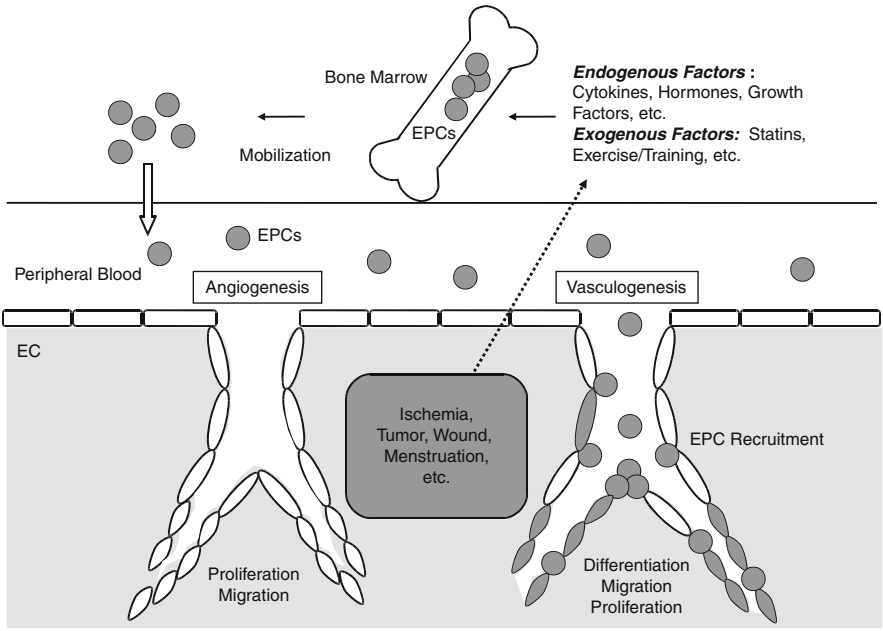


Fig. 35.1 The concept of "angiogenesis" shows that preexisting ECs proliferate and migrate to form a new vessel in response to the endogenous or exogenous stimuli (*left* in the figure). In contrast, a variety of factors released from the jeopardized tissue or surrounding area affect BM remotely and mobilize EPCs from the BM into circulation. EPCs recruit (home) to the site and participate in neovascularization differentiating, proliferating, and migrating, which is the concept of "vasculogenesis" (*right* in the figure)

arteriovenous vascular system that eventually forms following the onset of blood circulation (Risau et al. 1988). The relationship between the cells which circulate in the vascular system (hematopoietic stem cells, HSCs) and those responsible for the vessels themselves (EPCs/angioblasts) is determined by their spatial orientation in the fused blood islands; HSCs are located in the center of the blood islands versus EPCs/angioblasts are at the periphery. In addition to this arrangement, HSCs and EPCs share certain common antigens, including CD34, KDR, Tie-2, CD117, and Sca-1 (Choi et al. 1998).

Recently, EPCs were successfully isolated from circulating mononuclear cells (MNCs) in adults using KDR, CD34, and CD133 antigens shared by both embryonic EPCs and HSCs (Asahara et al. 1997; Peichev et al. 2000; Yin et al. 1997). *In vitro*, these cells differentiate into endothelial lineage cells, and in animal models of ischemia, heterologous, homologous, and autologous EPCs have been shown to recruit to the foci of angiogenesis contributing to neovascularization. Similar studies in which EPCs were isolated from human cord blood have also demonstrated their analogous differentiation into ECs *in vitro* and *in vivo* (Crisa et al. 1999; Kang et al. 2001; Murohara et al. 2000; Nieda et al. 1997). There is an emerging evidence that vasculogenesis does make a significant contribution to postnatal neovascularization.

Recent studies with animal BM transplantation (BMT) models, in which BM (donor)-derived EPCs could be traced, have shown that the contribution of EPCs to new vessel formation may range from 5 to 25% in response to granulation tissue formation (Crosby et al. 2000) or growth factor-induced neovascularization (Murayama et al. 2002). Also, in the tumor neovascularization, the range is approximately 35–45% higher than the former events (Reyes et al. 2002). The extent of EPC contribution to post-natal neovascularization depends on each neovascularizing event or disease.

Since the discovery of EPCs, (Asahara et al. 1997; Shi et al. 1998) other investigators have also tried to define this cell population. However, since EPCs and HSCs share a number of surface markers, it is difficult to define EPCs with certain simple markers. The term EPC may therefore encompass a heterogenous cell population including the cells in various stages ranging from hemangioblasts to fully differentiated ECs, even though a variety of methods of EPC isolation have been reported (Asahara et al. 1997; Boyer et al. 2000; Fernandez Pujol et al. 2000; Gehling et al. 2000; Gunsilius et al. 2000; Harraz et al. 2001; Kalka et al. 2000; Kang et al. 2001; Lin et al. 2000; Murohara et al. 2000; Nieda et al. 1997; Peichev et al. 2000; Quirici et al. 2001; Schatteman et al. 2000; Shi et al. 1998). Although the putative precursors of EPC and the differentiated final ECs remain to be determined, there is strong evidence *in vivo* that a population of EPCs exists in humans.

Lin et al. cultivated peripheral MNCs from patients receiving gender-mismatched BMT and studied their growth *in vitro*. In this study, they identified a population of BM (donor)-derived ECs with high proliferative potential (late outgrowth); these BM cells likely represent EPCs (Lin et al. 2000). Gunsilius et al. investigated a chronic myelogenous leukemia model and disclosed that BM-derived EPCs contribute to postnatal neovascularization in human (Gunsilius et al. 2000). Interestingly, BM-derived EPCs could be detected even in the wall of quiescent vessels without neovascularization events, suggesting that BM-derived EPCs may be related even to the turnover of ECs consisting of quiescent vessels. Reyes et al. have isolated multipotent adult progenitor cells (MAPCs) from BM MNCs and differentiated them into EPCs, indicating that MAPCs are an origin of EPCs (Reyes et al. 2002). These studies therefore provide evidence to support the presence of BM-derived EPCs that participate in neovascularization. However, the existence of namely '*in situ* EPCs' as derived from tissue specific stem/progenitor cells in murine skeletal muscle remains to be investigated even in the other tissues (Lin et al. 2000) (Fig. 35.1).

35.1.3 EPC Kinetics in Peripheral Blood

As described previously, tissue trauma causes mobilization of hematopoietic cells as well as pluripotent stem or progenitor cells from the hematopoietic system (Grzelak et al. 1998). Consistent with the notion that EPCs and HSCs share common

surface angigens, our recent data has shown that mobilization of BM-derived EPCs constitutes a natural response to tissue ischemia. The murine BMT model also provided direct evidence of enhanced BM-derived EPC incorporation into foci of corneal neovascularization following the development of hindlimb ischemia, (Takahashi et al. 1999) indicating that circulating EPCs are mobilized endogenously in response to tissue ischemia and can incorporate into neovascular foci to promote tissue repair. These results in animals were recently confirmed by human studies illustrating EPC mobilization in patients following burns, CABG, or acute myocardial infarction (Shintani et al. 2001).

In the pathophysiological events that require neovascularization *in vivo*, a variety of cytokines, growth factors, or hormones released from the jeopardized tissue affect BM remotely and cause EPC mobilization from BM. For instance, granulocyte macrophage colony-stimulating factor (GM-CSF) is well known to stimulate hematopoietic progenitor cells and myeloid lineage cells, but has recently been shown to exert a potent stimulatory effect on EPC kinetics. The delivery of this cytokine induced EPC mobilization and enhanced neovascularization in severely ischemic tissues and *de novo* corneal vascularization (Takahashi et al. 1999). Vascular endothelial growth factor (VEGF), critical for angio/vasculogenesis in the embryo, (Ferrara et al. 1996; Carmeliet et al. 1996; Shalaby et al. 1995) has also been shown to be an important stimulus of adult EPC kinetics recently. Our studies performed first in mice (Asahara et al. 1999b) and subsequently in patients undergoing VEGF gene transfer for limb or myocardial ischemia (Kalka et al. 2000) revealed a previously unappreciated mechanism by which VEGF contributes to neovascularization in part by mobilizing BM-derived EPCs. Similar modulation of EPC kinetics has been observed in response to other hematopoietic stimulators; granulocyte-colony stimulating factor (G-CSF) and stromal-derived factor-1 (SDF-1), (Moore et al. 2001) growth factors; platelet derived growth factor -CC (PDGF-CC), (Li et al. 2005) brain derived neurotropic factor (BDNF)(Kermani et al. 2005) and placental growth factor (PlGF), (Hattori et al. 2002) and hormones; estrogen (Iwakura et al. 2003) and erythropoietin (Heeschen et al. 2003) (Fig. 35.1). The distinct mechanism by which EPCs are mobilized to the peripheral circulation remains unknown, but may mimic aspects of embryonic development.

EPC mobilization has recently been implicated not only by natural hematopoietic or angiogenic stimulants but also by pharmacological agents. For instance, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are known to rapidly activate Akt signaling in ECs, thereby stimulating EC bioactivity *in vitro* and enhancing angiogenesis *in vivo* (Kureishi et al. 2000). Recent studies by Dimmeler *et al*. and our laboratory have demonstrated a novel function of statins by mobilizing BM-derived EPCs through the stimulation of the Akt signaling pathway (Vasa et al. 2001a, b; Urbich et al. 2002; Llevadot et al. 2001; Dimmeler et al. 2001). Therefore this newly appreciated role of statins, along with their already well-established safety and efficacy on hypercholesterolemia, suggests that they can be beneficial in treating various forms of vascular diseases.

35.2 Role of EPCs in Post-natal Neovascularization

35.2.1 Direct EPC Contribution to Neovascularization

Post-natal neovascularization was originally recognized to be constituted by the mechanism of "angiogenesis", which is new vessel formation, operated by *in situ* proliferation and migration of preexisting ECs as previously described (Folkman and Shing 1992). However, the discovery of EPCs resulted in the addition of the new mechanism for vascular formation in adults, "vasculogenesis", which is frequently observed during embryogenesis. "Vasculogenesis" is *de novo* vessel formation by *in situ* incorporation, differentiation, migration, and/or proliferation of BM-derived EPCs (Asahara et al. 1999a). The incorporation of BM-derived EPCs into foci of physiological and pathological neovascularization has been demonstrated in various animal experiments. One well-established model that allows us to detect BM-derived EPCs utilizes wild-type mice with BM cells transplanted from transgenic mice in which LacZ expresses under the regulation of an EC lineage-specific promoter, flk-1 or Tie-2 (Flk-1/LacZ/BMT, Tie-2/LacZ/BMT). Using these mice, Flk-1- or Tie-2-expressing endothelial lineage cells derived from BM (EPCs) have been shown to localize to vessels during tumor growth, wound healing, skeletal and cardiac ischemia, corneal neovascularization, and endometrial remodeling following hormone-induced ovulation (Asahara et al. 1999a) (Figs. 35.1 and 35.2). On the other hand, tissue specific stem/progenitor cells with the potency of differentiation into myocytes or ECs was also isolated in skeletal muscle tissue in murine hindlimb later on, although the origin of the cells remains to be cleared (Tamaki et al. 2002). This finding suggests that the origin of EPCs may not be limited to BM, e.g., tissue specific stem/progenitor cells possibly provide "*in situ* EPCs" as other sources of EPCs than BM. Regardless of the origin of EPCs, they certainly play a significant role contributing to neovascularization directly via vasculogenesis in the tissue.

35.2.2 Indirect EPC Contribution to Neovascularization

Apart from the established role of EPCs in neovascularization, namely "direct participation in neovasculature via vasculogenesis", recruited EPCs to the jeopardized tissue that requires vessel regeneration do not always participate in the neovasculature but rather stay in interstitial tissue along with neovascularization. (Fig. 35.2) These 'resting' EPCs in the tissue produce a variety of cytokines/growth factors, specifically pro-angiogenic ones, and promote pre-existing EC proliferation and migration resulting in angiogenesis. This paracrine effect of EPCs represents indirect contribution to neovascularization. As far as we and others confirm the cytokines/growth factors produced from EPCs, EPCs will release VEGF, hepatocyte

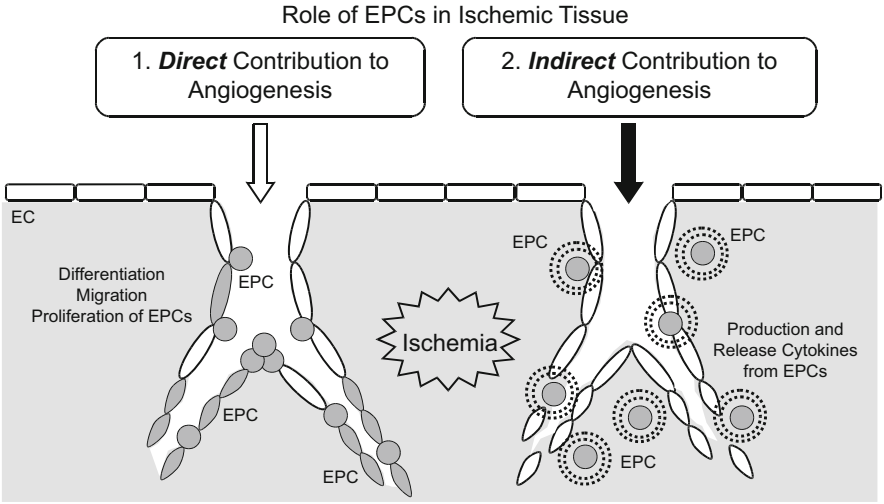


Fig. 35.2 Two different roles of EPCs in neovascularization. In the case of new vessel formation, one role of EPCs is the direct participation of EPCs in neovascularization accompanying preexisting EC proliferation and migration (*left* in the figure). The other role of EPCs is the indirect effect on angiogenesis with the production and release of pro-angiogenic cytokines/growth factors from recruited EPCs. These EPCs remain in the site without participating in the neovasculature, exhibiting so-called "paracrine effect" (*right* in the figure)

growth factor (HGF), angiopoietin-1 (Ang-1), endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), SDF-1α, and insulin-like growth factor-1 (IGF-1), etc. Both VEGF and HGF promote EC proliferation leading to angiogenesis, and Ang-1 may play a role for stabilizing pre-matured vessels in ischemic tissue. Nitric oxide (NO) synthase, by ether eNOS or iNOS, maintains tissue blood perfusion in microcirculating systems acting as a vasodilator. Since little eNOS expression is observed in cardiac capillaries except for ECs in coronary arteries, 'imported' eNOS produced from the recruited EPCs is thought to be a major source of eNOS and important in short term ischemia, specifically inchemia-reperfusion injury (Ii et al. 2005). iNOS is also produced from the recruited EPCs, however, the expression is prominent only when the tissue hypoxia is sustained for a long time, i.e. in the case of chronic myocardial ischemia rather than ischemia-reperfusion injury. BM-derived cell eNOS or iNOS deletion results in the exacerbation of myocardial infarction induced by ischemia-reperfusion injury or permanent vessel occlusion, respectively, suggesting that 'imported' NOS is crucial for preventing ischemic myocardium depending on the type of ischemic injury (Ii et al. 2005). SDF-1α released from recruited EPCs further recruits more EPCs triggering a chain reaction. On the other hand, EPCs will prevent cardiac apoptosis caused by ischemia via a production of IGF-1, a potent anti-apoptotic factor, activating the Akt

signaling pathway. Thus, EPCs demonstrate tissue-protective effects producing favorable factors, namely "indirect contribution to neovascularization in ischemic tissue".

35.3 EPC-Based Therapeutic Angiogenesis

Since the discovery of EPCs in 1997, we immediately focused on the regenerative potential of stem/progenitor cells as well as the unique characteristics. *In vitro*, stem/progenitor cells have the capability of self-renewal and differentiation into organ-specific cell types. *In vivo*, these cells are then directed by the appropriate milieu that allows them to differentiate and reconstitute target organs. The novel therapeutic strategy for ischemic diseases, EPC transplantation, may therefore be an epoch as a cell therapy involving the classic paradigm of angiogenesis developed by Folkman and colleagues.

35.3.1 EPC Transplantation in Experimental Animals

We and others indicated that cell therapy with culture-expanded EPCs can successfully promote neovascularization in ischemic tissue, even when administered as "sole therapy," i.e., in the absence of angiogenic growth factors. Such a "supply-side" version of therapeutic neovascularization in which the substrate (EPCs/ECs) rather than ligand (growth factor) comprises the therapeutic tool, was first reported by intravenously transplanting human EPCs to immunodeficient mice with hindlimb ischemia (Kalka et al. 2000). These findings provided a novel insight that exogenously administered EPCs restored impaired neovascularization in a mouse ischemic hindlimb model. A similar study in which human EPCs were transplanted in a myocardial ischemia model of nude rat, demonstrated that transplanted EPCs localized to the area of neovascularization with the differentiation into mature ECs. These findings were consistent with preserved left ventricular (LV) function and reduced infraction size (Kawamoto et al. 2001). Another study in which human cord blood-derived EPCs were transplanted in an ischemic hindlimb model of nude rats also demonstrated similar findings with enhanced neovascularization in ischemic tissue (Murohara et al. 2000) (Fig. 35.3).

Recently, other investigators have explored the therapeutic potential of CD34+ Cells as an EPC-enriched fraction. Shatteman et al. transplanted freshly isolated human CD34+ cells into diabetic nude mice with hindlimb ischemia, and showed a blood flow recovery in the ischemic limb (Schatteman et al. 2000). Also, Kocher et al. attempted intravenous infusion of freshly isolated human CD34+ cells into nude rats with myocardial ischemia, and observed preservation of LV function in consistent with the inhibition of cardiac apoptosis (Kocher et al. 2001). CD34+ cell dose-dependent contribution to LV functional recovery and neovascularization in ischemic myocardium has been demonstrated. Notably, CD34+ cells in higher dose groups committed into not only vasculogenic (endothelial and mural) lineage but also myocardial lineage cells (Iwasaki et al. 2006) (Fig. 35.3).

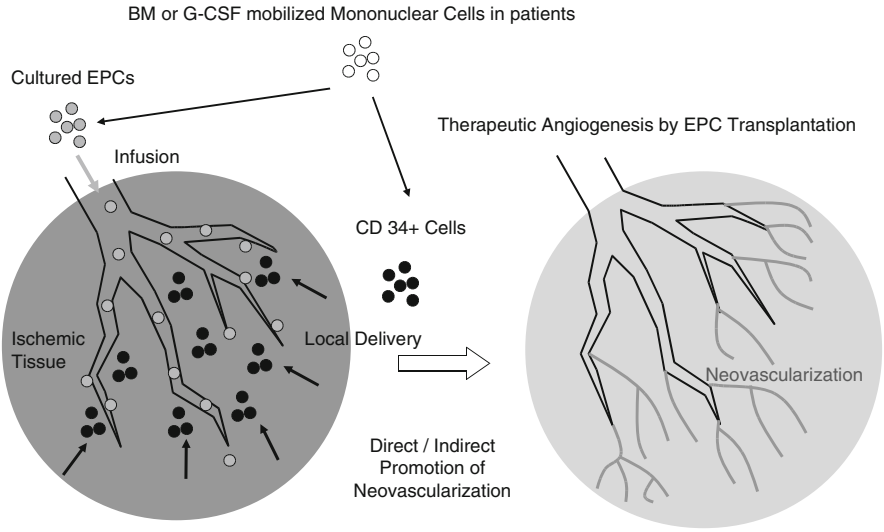


Fig. 35.3 Therapeutic angiogenesis/vasculogenesis with EPC transplantation. In clinical trials, both freshly isolated CD34+ cells from G-CSF mobilized mononuclear cells in peripheral blood of patients with chronic ischemic myocardial ischemia and cultured EPCs from bone marrow or peripheral blood in patients with acute myocardial infarction have been used. Indeed, these EPCs give rise to favorable outcomes regardless of the type of EPCs

35.3.2 EPC Transplantation in Clinical Trials

We have recently reported a phase I/II clinical trial regarding intramuscular transplantation of autologous and G-CSF-mobilized CD34+ cells in patients with intractable critical limb ischemia (CLI) (Kawamoto et al. 2009). The first-in man trial was conducted as a prospective, multicenter, single-blinded and dose-escalation study since 2003 in our institute. G-CSF was used to efficiently mobilize BM-EPCs to PB, and the mobilized CD34+ cells were isolated as EPC-enriched fraction.

In all subjects, primary endpoint, the Efficacy score at week 12 was positive value indicating improvement of lower limb ischemia after the cell therapy. In addition, both subjective and objective parameters of lower limb ischemia such as toe brachial pressure index (TBPI), transcutaneous partial oxygen pressure ($TcPO_2$), total walking distance (TWD), pain-free walking distance (PFWD), Wang-Baker's pain rating scale and the ulcer size significantly, (Fig. 35.4) and serially improved after transplantation of CD34+ cells. Because this was not a randomized, controlled study, possibility of the placebo effect after CD34+ cell transplantation needs to be evaluated in the large-scaled future trial. As for the safety evaluation, neither death nor life-threatening adverse events were observed in this study. No severe adverse event, for which relation to a series of cell therapy could not be denied, was also observed. Although mild to moderate adverse events were frequent, these events were transient and expected. No malignant tumor was also clinically identified during the study period.

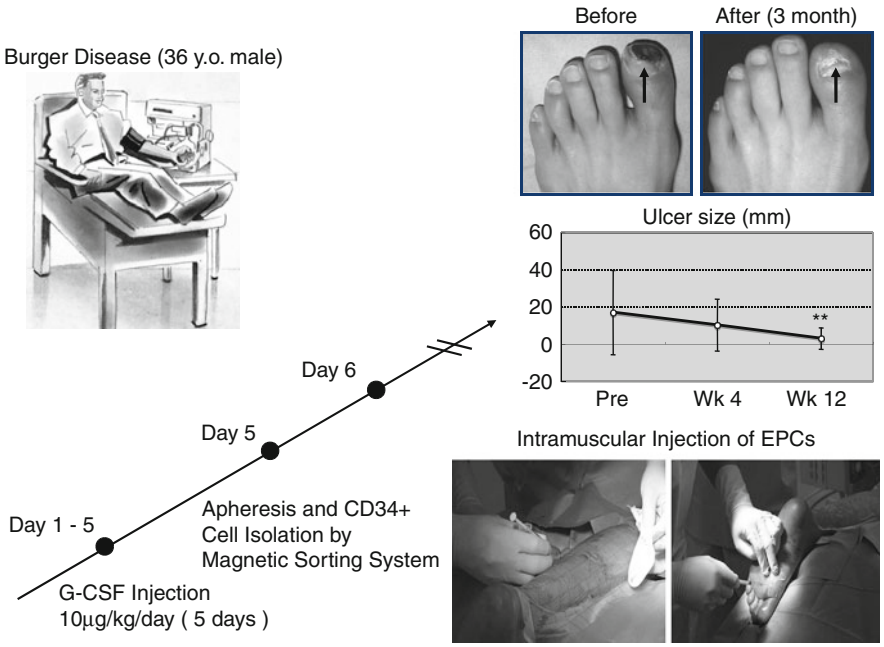


Fig. 35.4 Representative case of autologous CD34+ cell transplantation therapy for CLI in Burger disease. Thirty-six year old male patient who had toe necrosis due to microcirculation failure received CD34+ cell injection at 40 sites in ischemic limb under lumbar anesthesia, and the necrosis was significantly improved with blood flow recovery with reduced skin ulcer size 3 months after the treatment. Quantitative analysis for skin ulcer size exhibits significant improvement of toe necrosis (*Graph*). ** $P<0.01$ vs. Pre (*baseline*). The improvement could be maintained for more than 1 year without recurrence

In addition to CD34, CD133 is a surface marker of early EPC phenotype. A recent clinical study by Burt et al. showed the safety and feasibility of autologous, GCSF-mobilized CD133+ cell implantation into lower extremity muscles of nine patients with CLI including a patient with Buerger's disease (Burt et al. 2010). PB-MNCs were collected by leukapheresis after GCSF mobilization (10 μg/kg/day for 4–5 days), and CD133+ stem cells were selected using a magnetic separation system. There were no major complications from either leukapheresis or cell injection. The patient with Buerger's disease underwent the procedure twice. After the procedure, rest pain resolved rapidly by day 2, and 7 of 9 patients including a case of Buerger's disease were able to avoid limb amputation during the 1-year follow-up.

Although these studies were small-sized, non-randomized trials, these initial results suggest the potential effectiveness of the purified EPC population in CLI patients.

As for the EPC therapy for coronary artery disease, Losordo et al. recently reported a phase II, randomized, placebo-controlled and dose-ranging clinical trial for 167 patients with refractory angina. Autologous CD34+ cells isolated from GCF-mobilized apheresis products were intramyocardially injected into ischemic myocardium under

the guidance of NOGA endomyocardial mapping. Six and tweleve months later, angina counts and changes in exercise time significantly improved in CD34+ cell group than placebo group (Losordo et al. 2011). These promising outcomes also support the clinical usefulness of EPC transplantation for reduction of tissue ischemia.

35.3.3 Problems in EPC Transplantation

Our animal studies (Kalka et al. 2000) suggest that heterologous EPC transplantation requires systemic injection of $0.5 \sim 2.0 \times 10^4$ human EPCs/g body weight of the recipient animal to achieve satisfactory improvement of hindlimb ischemia. In general, cultured EPCs obtained from healthy human volunteers yields 5.0×10^6 cells per 100 ml of peripheral blood on day 7. Based on these data in human, a blood volume of as much as 12 l will be necessary to obtain enough number of EPCs to treat patients who have critical ischemic hindlimb. Therefore, the background factors in clinical patients such as aging, (Heiss et al. 2005) diabetes, (Ii et al. 2006; Vasa et al. 2001b) hypercholesterolemia, (Vasa et al. 2001a, b) hypertension (Vasa et al. 2001a, b; Imanishi et al. 2005) and smoking (Kondo et al. 2004; Michaud et al. 2006) that may reduce the number of circulating/BM EPCs and the function will cause major limitations of primary EPC transplantation. In reality, most of the patients who are going to undergo EPC therapy for the ischemic diseases more or less have background diseases as described above. Considering autologous EPC therapy, certain technical improvements that may help to overcome the malfunction of EPCs should include; (1) local delivery of EPCs, (2) endogenous EPC mobilization i.e. cytokine/growth factor supplements to promote BM-derived EPC mobilization, (Asahara et al. 1999b; Takahashi et al. 1999), (3) enrichment procedures, i.e., leukapheresis or BM aspiration, (4) enhancement of EPC functions by gene transduction, or (5) culture-expansion of EPCs from self-renewable primitive stem/progenitor cells in BM or other sources. Unless the quality and quantity of autologous EPCs is obtained by the technical improvements as described above, allogenic EPCs derived from umbilical cord blood or culture-expanded from human embryonic stem cells (Murohara et al. 2000; Levenberg et al. 2002), may be another alternative source supplying EPCs.

35.3.4 EPC as a Biomarker for Ischemic Diseases

Previous clinical studies reported that the number of circulating EPCs defined with cell surface markers, CD34+, CD34+/KDR+, CD133+/KDR, or CD34/CD133/KDR+, inversely correlated with the severity of cardiovascular diseases including congestive heart failure (Diller et al. 2008; Lev et al. 2005; Schmidt-Lucke et al. 2005; Shintani et al. 2001; Valgimigli et al. 2004; Werner et al. 2005) (Table 35.1). Colony forming activity of EPCs analyzed by Hill's method (Hill et al. 2003) has also been known to correlate with the number of circulating EPCs and used for the

Table 35.1 Correlation between circulating EPC number/function and cardiovascular diseases

Subject disease	Control disease	N	EPC type	EPC number/function	References
AMI (Day 7 after onset)	AMI (Day of onset)	16	CD34+	2X↑/CFA↑	Shintani et al. (2001)
Non-ST↑AMI (with collaterals)	Non-ST↑AMI (without collaterals)	20	CD133+/KDR+	2X↑/CFA →	Lev et al. (2005)
CAD (Cardiac event +)	CAD (Cardiac event −)	77	CD34+/KDR+	0.5X↓/ (~)	Schmidt-Lucke et al. (2005)
CAD (Cardiac event −)	CAD (Cardiac event +)	519	CD34+/KDR+	1.5–2X↑/ (~)	Werner et al. (2005)
Congestive HF	Healthy volunteer	46	CD34+/CD133+/KDR+	Mild HF:3–4X↑/CFA↑ Severe HF:0.7–0.5X倍↓/CFA↓	Valgimigli et al. (2004)
Eisenmenger Syndrome (with pulmonary HT)	Healthy volunteer	96	CD34+, CD34+/CD133+, KDR+	0.3–0.5X↓/CFA↓	Diller et al. (2008)

AMI acute myocardial infarction, *CAD* coronary artery disease, *HF* heart failure, *HT* hypertension, *CFA* colony forming activity

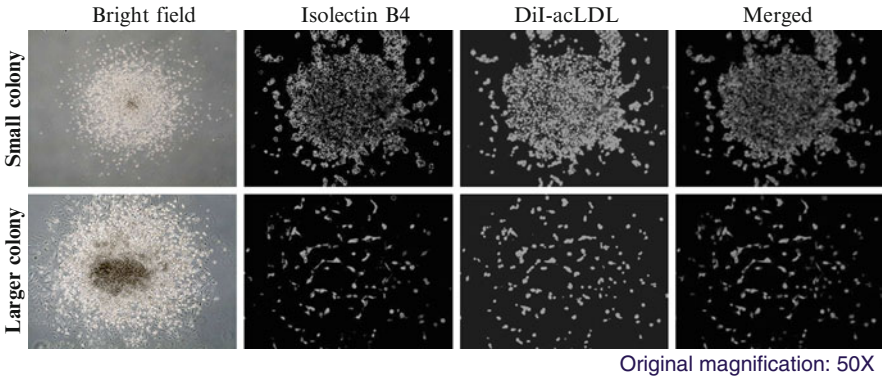


Fig. 35.5 Representative images of large EPC colony and small EPC colony with human CD34+ cells. GCS-F mobilized human CD34+ cells were cultured in methylcellulose-based special culture medium for 3 weeks and the formed colonies were identified as colonies consist of large EPCs and those consist of small EPCs according to the morphology-based criteria. Both small EPC colony (*upper panels*) and large EPC colony (*lower panels*) show characteristics of acLDL uptake and isolectin B4 binding

assessment of EPC function, however, Hill's colony assay is recognized as just a method for detecting EPC aggregation.

Thus, we have recently developed a novel EPC colony forming assay (EPC-CFA) system, capable to address and overcome most of the limitations of the classical assay systems, is challenging several of the predominant classical opinions about EPCs, and enabling an until now missing differential hierarchic view on EPCs. We have reported one of the first examples of such an assay system, initially designed to work with mouse samples. c-Kit$^+$/Sca-1$^+$/Lineage negative (KSL) cells were used as a putative murine hematopoietic EPC-enriched cell population, allowing the identification of two clearly distinguishable types of colonies (small and large colonies) that in turn correspond to two distinct EPC populations, primitive (small) and definitive (large) EPCs, respectively (Kwon et al. 2008; Tanaka et al. 2008; Kamei et al. 2010) (Fig. 35.5). The concept of an EPC-CFA was recently introduced and further developed for analysis of human EPC samples (Masuda et al. 2011). The EPC-CFA enables hereby not only the EPC-colony formation analysis of single and/or bulk cells from EPC-enriched arbitrary fractions or non-selected cell populations but allows also the cell fate analysis of primary and/or suspension culture cultivated single and/or bulk cells. It can further be easily combined with a classical HPC colony assay system, thus allowing a direct and comprehensive elucidation of the differences and similarities between EPCs and HPCs via the clarification of the cell fate of each cell type. The use of such an EPC-CFA allows not only the elucidation of a possible but so far elusive differentiation hierarchy of EPCs, but can be further used to identify and characterize the parameters associated with proliferation, commitment, and differentiation of EPCs *in vitro* and *in vivo* (Asahara et al. 2011).

Indeed, application of EPC-CFA on human CD34+ or CD133+ stem/progenitor cells enabled the identification of small and large distinct colony types each derived from a single cell, small-EPCs and large-EPCs, respectively. Small-EPCs showed a higher rate of proliferative activity with a higher number of cells being in the S-phase, when compared to large-EPCs. Interestingly, large-EPCs showed a significantly higher rate of vasculogenic activity with overall increased potential for cell adhesion and tube-like structure formation *in vitro* as well as a high *in vivo de novo* blood vessel forming activity following transplantation of these cells into a murine ischemic hindlimb model, as compared to small-EPCs. In contrast to small-EPCs, large-EPCs did not form secondary colonies but gave rise to isolated endothelial cell (EC) like cells when reseeded. Due to the observed *in vitro* (by FACS analysis) and *in vivo* characteristics of these colony types, small-EPCs were further characterized and believed to represent "primitive EPCs", a highly immature and proliferative population of cells, compared to large-EPCs which are believed to represent "definitive EPCs", cells prone to differentiate and promote vasculogenesis.

The advantage of these assessment for the number and colony forming activity of circulating EPCs is a convenient tool for clinical application in terms of a medical regulatory feasibility of sampling from blood cells by antibody targeting isolation, and a potent effectiveness on ischemic diseases through vasculogenic and angiogenic mechanisms by primary cells.

35.3.5 Future Strategy with EPC Transplantation

The possible and feasible strategy that may recover potential EPC dysfunction in ischemic disorders should be considered, given the findings that EPC function and mobilization may be impaired in certain diseases. One of the strategies, genetic modification of EPCs to overexpress angiogenic growth factors, will enhance signaling activity of the angiogenic response and reactivate the bioactivity and/or extend the life span of EPCs.

We have recently shown for the first time that gene-modified EPCs rescue impaired neovascularization in an animal model of limb ischemia (Iwaguro et al. 2002). Transplantation of heterologous EPCs transduced with adenovirus encoding human VEGF165 improved neovascularization and blood flow recovery, reducing the limb necrosis and auto-amputation rate in comparison with controls. The dose of EPCs needed to achieve limb salvage in these *in vivo* experiments was 30 times less than that required in the previous experiments involving unmodified EPCs (Kalka et al. 2000). Other investigators have also demonstrated the therapeutic efficacy of genetically engineered EPCs with a variety of target genes such as adrenomedullin (AM), (Nagaya et al. 2003) eNOS, (Kong et al. 2004) tissue plasminogen activator (tPA) (Griese et al. 2003) and integrin-like kinase (ILK) (Cho et al. 2005) in animal models. Thus, genetic modification might overcome the potential problems in the patients' EPCs for EPC transplantation therapy in ischemic diseases as so-called "second generation EPC therapy". Also, combining EPC

cell therapy with gene (i.e., VEGF) therapy (Kawamoto et al. 2004) may be another option to address the limited number and function of EPCs that can be isolated from peripheral blood in patients.

35.4 Summary

There is accumulating evidence that BM-derived EPCs have characteristics similar to those of angioblasts demonstrating the potential to promote postnatal vasculogenesis in adults, and clinical applications of EPCs in regenerative medicine are now on going. To acquire optimal quality and quantity of EPCs, however, several issues remain to be addressed, such as the development of a more efficient method of EPC purification and expansion, the methods of administration, and background disease-induced dysfunction or senescence in EPCs in patients. Alternatively, in the case of impossible utility of autologous BM-derived EPCs in patients with impaired BM function, appreciable EPCs isolated from umbilical cord blood or differentiated from tissue specific stem/progenitor or embryonic stem cells need to be optimized for EPC therapy. However, the unlimited potential of EPCs along with the emerging concepts of autologous cell therapy with gene modification suggests that they may soon reach clinical fruition.

References

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275(5302):964–967

Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM (1999a) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 85(3):221–228

Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM (1999b) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 18(14):3964–3972. doi:10.1093/emboj/18.14.3964

Asahara T, Kawamoto A, Masuda H (2011) Concise review: circulating endothelial progenitor cells for vascular medicine. Stem Cells 29(11):1650–1655. doi:10.1002/stem.745

Boyer M, Townsend LE, Vogel LM, Falk J, Reitz-Vick D, Trevor KT, Villalba M, Bendick PJ, Glover JL (2000) Isolation of endothelial cells and their progenitor cells from human peripheral blood. J Vasc Surg 31(1 Pt 1):181–189. doi:S0741521400696751 [pii]

Burt RK, Testori A, Oyama Y, Rodriguez HE, Yaung K, Villa M, Bucha JM, Milanetti F, Sheehan J, Rajamannan N, Pearce WH (2010) Autologous peripheral blood CD133+ cell implantation for limb salvage in patients with critical limb ischemia. Bone Marrow Transplant 45(1):111–116. doi:bmt2009102 [pii] 10.1038/bmt.2009.102

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380(6573):435–439

Cho HJ, Youn SW, Cheon SI, Kim TY, Hur J, Zhang SY, Lee SP, Park KW, Lee MM, Choi YS, Park YB, Kim HS (2005) Regulation of endothelial cell and endothelial progenitor cell survival and vasculogenesis by integrin-linked kinase. Arterioscler Thromb Vasc Biol 25(6):1154–1160. doi:01.ATV.0000164312.20008.93 [pii] 10.1161/01.ATV.0000164312.20008.93

Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G (1998) A common precursor for hematopoietic and endothelial cells. Development 125(4):725–732

Crisa L, Cirulli V, Smith KA, Ellisman MH, Torbett BE, Salomon DR (1999) Human cord blood progenitors sustain thymic T-cell development and a novel form of angiogenesis. Blood 94(11):3928–3940

Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Seifert RA, Bowen-Pope DF (2000) Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. Circ Res 87(9):728–730

Diller GP, van Eijl S, Okonko DO, Howard LS, Ali O, Thum T, Wort SJ, Bedard E, Gibbs JS, Bauersachs J, Hobbs AJ, Wilkins MR, Gatzoulis MA, Wharton J (2008) Circulating endothelial progenitor cells in patients with Eisenmenger syndrome and idiopathic pulmonary arterial hypertension. Circulation 117(23):3020–3030. doi:CIRCULATIONAHA.108.769646 [pii] 10.1161/CIRCULATIONAHA.108.769646

Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H, Zeiher AM (2001) HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J Clin Invest 108(3):391–397. doi:10.1172/JCI13152

Fernandez Pujol B, Lucibello FC, Gehling UM, Lindemann K, Weidner N, Zuzarte ML, Adamkiewicz J, Elsasser HP, Muller R, Havemann K (2000) Endothelial-like cells derived from human CD14 positive monocytes. Differentiation 65(5):287–300

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380(6573):439–442

Folkman J, Shing Y (1992) Angiogenesis. J Biol Chem 267:10931–10934

Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK, Fiedler W (2000) In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 95(10):3106–3112

Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, Rafii S (2001) Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res 88(2):167–174

Griese DP, Achatz S, Batzlsperger CA, Strauch UG, Grumbeck B, Weil J, Riegger GA (2003) Vascular gene delivery of anticoagulants by transplantation of retrovirally-transduced endothelial progenitor cells. Cardiovasc Res 58(2):469–477. doi:S0008636303002669 [pii]

Grzelak I, Olszewski WL, Zaleska M, Ziolkowska A, Durlik M, Lagiewska B, Muszynski M, Rowinski W (1998) Surgical trauma evokes a rise in the frequency of hematopoietic progenitor cells and cytokine levels in blood circulation. Eur Surg Res 30(3):198–204

Gunsilius E, Duba HC, Petzer AL, Kahler CM, Grunewald K, Stockhammer G, Gabl C, Dirnhofer S, Clausen J, Gastl G (2000) Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. Lancet 355(9216):1688–1691. doi:S0140-6736(00)02241-8 [pii] 10.1016/S0140-6736(00)02241-8

Harraz M, Jiao C, Hanlon HD, Hartley RS, Schatteman GC (2001) CD34- blood-derived human endothelial cell progenitors. Stem Cells 19(4):304–312

Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, Zhu Z, Bohlen P, Witte L, Hendrikx J, Hackett NR, Crystal RG, Moore MA, Werb Z, Lyden D, Rafii S (2002) Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nat Med 8(8):841–849. doi:10.1038/nm740

Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S (2003) Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood 102(4):1340–1346. doi:10.1182/blood-2003-01-0223 2003-01-0223 [pii]

Heiss C, Keymel S, Niesler U, Ziemann J, Kelm M, Kalka C (2005) Impaired progenitor cell activity in age-related endothelial dysfunction. J Am Coll Cardiol 45(9):1441–1448. doi:S0735-1097(05)00357-8 [pii] 10.1016/j.jacc.2004.12.074

Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T (2003) Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 348(7):593–600. doi:10.1056/NEJMoa022287 348/7/593 [pii]

Ii M, Nishimura H, Iwakura A, Wecker A, Eaton E, Asahara T, Losordo DW (2005) Endothelial progenitor cells are rapidly recruited to myocardium and mediate protective effect of ischemic preconditioning via "imported" nitric oxide synthase activity. Circulation 111(9):1114–1120. doi:01.CIR.0000157144.24888.7E [pii] 10.1161/01.CIR.0000157144.24888.7E

Ii M, Takenaka H, Asai J, Ibusuki K, Mizukami Y, Maruyama K, Yoon YS, Wecker A, Luedemann C, Eaton E, Silver M, Thorne T, Losordo DW (2006) Endothelial progenitor thrombospondin-1 mediates diabetes-induced delay in reendothelialization following arterial injury. Circ Res 98(5):697–704. doi:01.RES.0000209948.50943.ea [pii] 10.1161/01.RES.0000209948.50943.ea

Imanishi T, Moriwaki C, Hano T, Nishio I (2005) Endothelial progenitor cell senescence is accelerated in both experimental hypertensive rats and patients with essential hypertension. J Hypertens 23(10):1831–1837. doi:00004872-200510000-00014 [pii]

Iwaguro H, Yamaguchi J, Kalka C, Murasawa S, Masuda H, Hayashi S, Silver M, Li T, Isner JM, Asahara T (2002) Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation 105(6):732–738

Iwakura A, Luedemann C, Shastry S, Hanley A, Kearney M, Aikawa R, Isner JM, Asahara T, Losordo DW (2003) Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. Circulation 108(25):3115–3121. doi:10.1161/01.CIR.0000106906.56972.83 01.CIR.0000106906.56972.83 [pii]

Iwasaki H, Kawamoto A, Ishikawa M, Oyamada A, Nakamori S, Nishimura H, Sadamoto K, Horii M, Matsumoto T, Murasawa S, Shibata T, Suehiro S, Asahara T (2006) Dose-dependent contribution of CD34-positive cell transplantation to concurrent vasculogenesis and cardiomyogenesis for functional regenerative recovery after myocardial infarction. Circulation 113(10):1311–1325

Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T (2000) Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci USA 97(7):3422–3427. doi:10.1073/pnas.070046397070046397 [pii]

Kamei N, Kwon SM, Alev C, Ishikawa M, Yokoyama A, Nakanishi K, Yamada K, Horii M, Nishimura H, Takaki S, Kawamoto A, Ii M, Akimaru H, Tanaka N, Nishikawa S, Ochi M, Asahara T (2010) Lnk deletion reinforces the function of bone marrow progenitors in promoting neovascularization and astrogliosis following spinal cord injury. Stem Cells 28(2):365–375. doi:10.1002/stem.243

Kang HJ, Kim SC, Kim YJ, Kim CW, Kim JG, Ahn HS, Park SI, Jung MH, Choi BC, Kimm K (2001) Short-term phytohaemagglutinin-activated mononuclear cells induce endothelial progenitor cells from cord blood CD34+ cells. Br J Haematol 113(4):962–969. doi:bjh2694 [pii]

Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, Silver M, Ma H, Kearney M, Isner JM, Asahara T (2001) Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 103(5):634–637

Kawamoto A, Murayama T, Kusano K, Ii M, Tkebuchava T, Shintani S, Iwakura A, Johnson I, von Samson P, Hanley A, Gavin M, Curry C, Silver M, Ma H, Kearney M, Losordo DW (2004) Synergistic effect of bone marrow mobilization and vascular endothelial growth factor-2 gene therapy in myocardial ischemia. Circulation 110(11):1398–1405. doi:10.1161/01.CIR.0000141563.71410.64 01.CIR.0000141563.71410.64 [pii]

Kawamoto A, Katayama M, Handa N, Kinoshita M, Takano H, Horii M, Sadamoto K, Yokoyama A, Yamanaka T, Onodera R, Kuroda A, Baba R, Kaneko Y, Tsukie T, Kurimoto Y, Okada Y, Kihara Y, Morioka S, Fukushima M, Asahara T (2009) Intramuscular transplantation of G-CSF-mobilized CD34(+) cells in patients with critical limb ischemia: a phase I/IIa, multicenter, single-blinded, dose-escalation clinical trial. Stem Cells 27(11):2857–2864. doi:10.1002/stem.207

Kermani P, Rafii D, Jin DK, Whitlock P, Schaffer W, Chiang A, Vincent L, Friedrich M, Shido K, Hackett NR, Crystal RG, Rafii S, Hempstead BL (2005) Neurotrophins promote revascularization by local recruitment of TrkB + endothelial cells and systemic mobilization of hematopoietic progenitors. J Clin Invest 115(3):653–663

Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S (2001) Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 7(4):430–436. doi:10.1038/86498 86498 [pii]

Kondo T, Hayashi M, Takeshita K, Numaguchi Y, Kobayashi K, Iino S, Inden Y, Murohara T (2004) Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. Arterioscler Thromb Vasc Biol 24(8):1442–1447. doi:10.1161/01.ATV.0000135655.52088.c5 01.ATV.0000135655.52088.c5 [pii]

Kong D, Melo LG, Gnecchi M, Zhang L, Mostoslavsky G, Liew CC, Pratt RE, Dzau VJ (2004) Cytokine-induced mobilization of circulating endothelial progenitor cells enhances repair of injured arteries. Circulation 110(14):2039–2046. doi:10.1161/01.CIR.0000143161.01901.BD 01.CIR.0000143161.01901.BD [pii]

Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, Walsh K (2000) The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med 6(9):1004–1010

Kwon SM, Eguchi M, Wada M, Iwami Y, Hozumi K, Iwaguro H, Masuda H, Kawamoto A, Asahara T (2008) Specific Jagged-1 signal from bone marrow microenvironment is required for endothelial progenitor cell development for neovascularization. Circulation 118(2):157–165. doi:10.1161/CIRCULATIONAHA.107.754978 CIRCULATIONAHA.107.754978 [pii]

Lev EI, Kleiman NS, Birnbaum Y, Harris D, Korbling M, Estrov Z (2005) Circulating endothelial progenitor cells and coronary collaterals in patients with non-ST segment elevation myocardial infarction. J Vasc Res 42(5):408–414. doi:JVR2005042005408 [pii] 10.1159/000087370

Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R (2002) Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci USA 99(7):4391–4396

Li X, Tjwa M, Moons L, Fons P, Noel A, Ny A, Zhou JM, Lennartsson J, Li H, Luttun A, Ponten A, Devy L, Bouche A, Oh H, Manderveld A, Blacher S, Communi D, Savi P, Bono F, Dewerchin M, Foidart JM, Autiero M, Herbert JM, Collen D, Heldin CH, Eriksson U, Carmeliet P (2005) Revascularization of ischemic tissues by PDGF-CC via effects on endothelial cells and their progenitors. J Clin Invest 115(1):118–127. doi:10.1172/JCI19189

Lin Y, Weisdorf DJ, Solovey A, Hebbel RP (2000) Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 105(1):71–77

Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, Walsh K, Isner JM, Asahara T (2001) HMG-CoA reductase inhibitor mobilizes bone marrow–derived endothelial progenitor cells. J Clin Invest 108(3):399–405. doi:10.1172/JCI13131

Losordo DW, Henry TD, Davidson C, Sup Lee J, Costa MA, Bass T, Mendelsohn F, Fortuin FD, Pepine CJ, Traverse JH, Amrani D, Ewenstein BM, Riedel N, Story K, Barker K, Povsic TJ, Harrington RA, Schatz RA (2011) Intramyocardial, autologous CD34+ cell therapy for refractory angina. Circ Res 109(4):428–436. doi:CIRCRESAHA.111.245993 [pii] 10.1161/CIRCRESAHA.111.245993

Masuda H, Alev C, Akimaru H, Ito R, Shizuno T, Kobori M, Horii M, Ishihara T, Isobe K, Isozaki M, Itoh J, Itoh Y, Okada Y, McIntyre BA, Kato S, Asahara T (2011) Methodological development of a clonogenic assay to determine endothelial progenitor cell potential. Circ Res 109(1):20–37. doi:10.1161/CIRCRESAHA.110.231837

Michaud SE, Dussault S, Haddad P, Groleau J, Rivard A (2006) Circulating endothelial progenitor cells from healthy smokers exhibit impaired functional activities. Atherosclerosis 187(2):423–432

Moore MA, Hattori K, Heissig B, Shieh JH, Dias S, Crystal RG, Rafii S (2001) Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. Ann N Y Acad Sci 938:36–45; discussion 45–37

Murayama T, Tepper OM, Silver M, Ma H, Losordo DW, Isner JM, Asahara T, Kalka C (2002) Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo. Exp Hematol 30(8):967–972. doi:S0301472X02008676 [pii]

Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, Onitsuka I, Matsui K, Imaizumi T (2000) Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 105(11):1527–1536. doi:10.1172/JCI8296

Nagaya N, Kangawa K, Kanda M, Uematsu M, Horio T, Fukuyama N, Hino J, Harada-Shiba M, Okumura H, Tabata Y, Mochizuki N, Chiba Y, Nishioka K, Miyatake K, Asahara T, Hara H, Mori H (2003) Hybrid cell-gene therapy for pulmonary hypertension based on phagocytosing action of endothelial progenitor cells. Circulation 108(7):889–895. doi:10.1161/01.CIR.0000079161.56080.22 01.CIR.0000079161.56080.22 [pii]

Nieda M, Nicol A, Denning-Kendall P, Sweetenham J, Bradley B, Hows J (1997) Endothelial cell precursors are normal components of human umbilical cord blood. Br J Haematol 98(3):775–777

Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S (2000) Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 95(3):952–958

Quirici N, Soligo D, Caneva L, Servida F, Bossolasco P, Deliliers GL (2001) Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells. Br J Haematol 115(1):186–194. doi:3077 [pii]

Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM (2002) Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 109(3):337–346. doi:10.1172/JCI14327

Risau W, Flamme I (1995) Vasculogenesis. Annu Rev Cell Dev Biol 11:73–91

Risau W, Sariola H, Zerwes HG, Sasse J, Ekblom P, Kemler R, Doetschman T (1988) Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. Development 102(3):471–478

Schatteman GC, Hanlon HD, Jiao C, Dodds SG, Christy BA (2000) Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. J Clin Invest 106(4):571–578. doi:10.1172/JCI9087

Schmidt-Lucke C, Rossig L, Fichtlscherer S, Vasa M, Britten M, Kamper U, Dimmeler S, Zeiher AM (2005) Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. Circulation 111(22):2981–2987. doi:CIRCULATIONAHA.104.504340 [pii] 10.1161/CIRCULATIONAHA.104.504340

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376(6535):62–66

Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MA, Storb RF, Hammond WP (1998) Evidence for circulating bone marrow-derived endothelial cells. Blood 92(2):362–367

Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y, Imaizumi T (2001) Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation 103(23):2776–2779

Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T (1999) Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 5(4):434–438. doi:10.1038/7434

Tamaki T, Akatsuka A, Ando K, Nakamura Y, Matsuzawa H, Hotta T, Roy RR, Edgerton VR (2002) Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle. J Cell Biol 157(4):571–577. doi:10.1083/jcb.200112106 jcb.200112106 [pii]

Tanaka R, Wada M, Kwon SM, Masuda H, Carr J, Ito R, Miyasaka M, Warren SM, Asahara T, Tepper OM (2008) The effects of flap ischemia on normal and diabetic progenitor cell function. Plast Reconstr Surg 121(6):1929–1942. doi:10.1097/PRS.0b013e3181715218 00006534-200806000-00007 [pii]

Urbich C, Dernbach E, Zeiher AM, Dimmeler S (2002) Double-edged role of statins in angiogenesis signaling. Circ Res 90(6):737–744

Valgimigli M, Rigolin GM, Fucili A, Porta MD, Soukhomovskaia O, Malagutti P, Bugli AM, Bragotti LZ, Francolini G, Mauro E, Castoldi G, Ferrari R (2004) CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. Circulation 110(10):1209–1212. doi:10.1161/01.CIR.0000136813.89036.21 01.CIR.0000136813.89036.21 [pii]

Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, Dimmeler S (2001a) Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 103(24):2885–2890

Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM, Dimmeler S (2001b) Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 89(1):E1–E7

Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Bohm M, Nickenig G (2005) Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 353(10):999–1007. doi:353/10/999 [pii] 10.1056/NEJMoa043814

Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, Olweus J, Kearney J, Buck DW (1997) AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 90(12):5002–5012

Chapter 36
Heart

Gustav Steinhoff and Bodo Eckehard Strauer

Abstract Coronary heart disease and chronic heart failure are common and have an increasing frequency. Although revascularisation procedures and conventional drug therapy may delay ventricular remodelling, there is no basic therapeutic regime available for preventing or even reversing this process. Chronic coronary artery disease and heart failure impair quality of life and are associated with subsequent worsening of the cardiac pump function. Numerous studies within the past few years have been demonstrated, that cardiac stem cell therapy has to be considered a safe therapeutic procedure in heart disease, when destroyed and/or compromised heart muscle must be regenerated. Different autologous or allogenic progenitor cell populations have been addressed for cardiac cell therapy. This kind of cell therapy with autologous bone marrow cells is completely justified ethical, except for the small numbers of patients with direct or indirect bone marrow disease (e.g. myeloma, leukaemic infiltration) in whom there would be intrinsic lesions of mononuclear cells. Several preclinical as well as clinical trials have shown that transplantation of autologous bone marrow stem cells or precursor cells improve cardiac function after myocardial infarction and in chronic ischemic heart disease. Further indications are non-ischemic and diabetic cardiomyopathy (dilated cardiomyopathy), as well as heart failure due to an infectious cause like Chagas heart disease. Other clinical applications in heart valve and vascular conduit tissue engineering have been approached. Further clinical development is aimed to modify cardiac inflammation and cardiogenesis by stem cell modification and to test other stem cell sources.

G. Steinhoff, M.D., Ph.D. (✉)
Department of Cardiac Surgery and Reference and Translation Center
of Cardiac Stem Cell Therapy (RTC), Medical Faculty, University of Rostock,
Schillingallee 35, 18057 Rostock, Germany
e-mail: gustav.steinhoff@med.uni-rostock.de

B.E. Strauer, M.D., FESC, FACC
Department of Cardiology, Pneumology and Angiology, Heinrich-Heine-University
of Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_36,

36.1 Background and Rationale for Stem Cell Therapy in Heart Disease

Stem cells have the important properties of self-regeneration and differentiational plasticity (Allgöwer 1956; Jiang et al. 2002; Krause et al. 2001; Quaini et al. 2004). Thus, they are ideal candidates for regeneration of damaged myocardial tissue (Goodell et al. 2001),for example, in myocardial infarction or congestive heart failure. When acute myocardial infarction occurs, heart muscle tissue is regionally destroyed (Pfeffer and Braunwald 1990; Ren et al. 2002). By percutaneous coronary interventions, coronary restitution can be achieved and coronary perfusion may be normalized by reopening coronary artery occlusions; however, regular heart muscle function may be restored not or only to a minor degree due to postischemic destruction, so that remodelling and heart failure mostly are not prevented. Prevention of remodelling which occurs in approximately 60% of postinfarction patients, however, may possibly be realized by cell transplantation, which leads to myocardial restitution with the beneficial aim of restoring or normalizing compromised heart function (Kocher et al. 2001).

One possible way of heart muscle repair is to transplant cells of primarily non-cardiac origin for cardiac regeneration. First clinical attempts to cardiac cell therapy using non-cardiac cells were made by the Paris-group (Menasche et al. 2001), who for the first time in the year 2000 applied autologous cultured skeletal myoblasts for intramyocardial injection in postinfarction patients during coronary bypass surgery. The origin of skeletal myoblasts from bone marrow stem cells was described by Ferrari et al. (1998). Bone marrow contains progenitor cells for different lineages including the cardiovascular system (Reyes et al. 2001). Bone marrow stem cells may also operate as a source of cardiac cells; that is, as precursor of heart muscle tissue and of coronary blood vessel cells as demonstrated by Orlic et al. (2001) and Kocher et al. (2001). Human bone marrow contains e.g. CD34/CD133-positive haematopoietic and CD34/CD133-negative mesenchymal stem cells (Reyes et al. 2001) and both these types of stem cells may contribute to heart muscle repair. The first steps and experimental cornerstones demonstrating mononuclear bone marrow stem cell differentiation to muscle cells in experimental heart disease, especially in experimental myocardial infarction, were realized by different investigators: demonstrating (i) regeneration of myocardium after infarction (Kocher et al. 2001) (ii) reduction of infarct size (Kocher et al. 2001); and (iii) de novo expression of cardiac proteins by human bone marrow cells (Toma et al. 2002). The Düsseldorf group performed clinical intravascular stem cell therapy for the first time in March 2001, when treating acute myocardial infarction by intracoronary cell transfer (Strauer et al. 2001). In July 2001 the Rostock group treated chronic ischemia after myocardial infarction in bypass surgery patients for the first time by intramyocardial injection of purified stem cell transplantation (Stamm et al. 2003). The aims of these procedures, which had not been achieved before, were:

1. To use the body's own (autologous) stem cells from bone marrow or from peripheral blood for cardiac tissue repair; that is, (a) for intracoronary (endovascular) application to use all three fractions of mononuclear bone marrow stem cells,

haematopoietic, angioblastic and mesenchymal; (b) for direct intramyocardial (interstitial) transplantation to use highly purified (f.e. CD133+) bone marrow stem cells.
2. To facilitate and potentiate cell migration by artificial cardiac ischaemia, which is one of the most effective stimuli for stem cell differentiation and recruitment (Kamota et al. 2009), most probably via the CXCR4 – SDF-1 interrelationships. In the endovascular approach this has been achieved by repetitive intracoronary balloon dilatations within the occlusion of the former infarct-related artery. In the intramyocardial approach this has been achieved by cardioplegic ischemia during the heart surgery procedure prior to stem cell application.
3. To enrich and to accumulate bone marrow stem cells within the postischemic infarct border zone either by intracoronary (endovascular) or intramyocardial (interstitial) administration.

In the recent past new evidence suggests that cardiomyocyte renewal takes place in humans, although this process seems to be of low magnitude (Bergmann et al. 2009). Future therapeutic strategies should be aimed to stimulate this process. Stem cell transplantation could offer a therapeutic tool to fully exploit this self-renewing capacity of the adult human heart.

36.2 Cell Types

36.2.1 Adult Tissue Derived Cells

36.2.1.1 Bone Marrow Hematopoetic Progenitor/Stem Cells

Bone marrow derived haematopoietic stem cells or circulating peripheral blood progenitor cells have been shown to differentiate into cardiomyocytes in culture making them particularly interesting for the treatment of cardiac disease since they represent a well characterised and ample source of progenitor cells (Joggerst and Hatzopoulos 2009). The isolation and the systemic delivery of bone marrow stem cells has been established before in the treatment of hematopoietic diseases. Surface markers characterising hematopoietic stem cells in the adult human bone marrow include CD 133, CD 34, and CD 117 (c-kit). Besides the adult bone marrow stem cells expressing hematopoietic markers can also be obtained from the umbilical cord peripheral blood and the placenta. Large scale clinical trials using bone marrow derived hematopoietic stem cells could proof the safety and feasibility of this cell population for cardiac transplantation and showed moreover a modest improvement of the left ventricular function and a significant reduction in subsequent cardiovascular events. Although experimental data suggests that for bone marrow derived stem cell with hematopoietic markers cardiomyocyte differentiation is comparatively rare (Nygren et al. 2004), transplantation of bone marrow derived hematopoietic stem cells is closely related with myocardial regeneration most likely due to neovascularisation and reduction of apoptosis (Ma et al. 2006; Tse et al. 2007).

36.2.1.2 Endothelial Progenitor Cells

Another cell type derived from the adult bone marrow, the endothelial progenitor cell (EPC), has been very promising in the recent past in order to become a potential therapeutic tool. Neoangiogenesis was once thought to occur only through the proliferation of mature endothelial cells in the injured region. This understanding was challenged by the discovery that EPC derived from the bone marrow home to sites of injury and incorporate into the microvasculature (Asahara et al. 1997; Shi et al. 1998). Although there is some controverse discussion about their true definition as a subpopulation of bone marrow stem cells, EPC's can be identified by their capability to acquire endothelial cell characteristics in vitro and in vivo. They express the surface markers such as CD 133, the vascular endothelial growth factor receptor 2 (VEGFR- 2), CD 34 and vascular endothelial cadherin (VE-cadherin) of which CD 133 and 34 are shared with hematopoietic stem cells. They are mobilised from the bone marrow in the state of injury like vascular trauma or myocardial infarction (Gill et al. 2001; Shintani et al. 2001). Besides their role in vascular repair there is also evidence that EPC can differentiate in cardiomyocytes (Iwasaki et al. 2006). In preclinical settings injection of EPC's into the infarcted myocardium improved left ventricular function and reduced fibrosis (Jujo et al. 2008; Kocher et al. 2001). These results led to small clinical trials assessing feasibility and safety. Moreover EPC's have already found a niche in the field of interventional cardiology, where coronary stents have been coated with anti CD 34-antibodies to trap circulating endothelial progenitors in order to prevent in-stent restenosis by augmenting the endothelialisation process (Aoki et al. 2005).

36.2.1.3 Mesenchymal Stem Cells

Mesenchymal stem cells (MSC) are a subset of stem cells that are located in the stroma of the bone marrow and can differentiate into osteoblasts, chondrocytes and adipocytes (Alhadlaq and Mao 2004; Jiang et al. 2002). They can be separated from haematopoietic stem cells by their ability to adhere to a culture dish (Alhadlaq and Mao 2004). MSC can also be induced to differentiate in vitro into cardiomyocytes (Makino et al. 1999). MSC are potentially advantageous as they seem to be less immunogenic than other cell lines(Dai et al. 2005). Preclinical animal studies with myocardial infarction models demonstrated improved left ventricular function and a reduction of infarct size(Amado et al. 2005; Dai et al. 2005). Difficulties however, may arise because of the remaining heterogeneity among MSC populations regarding their differentiation capacity and consecutive low predictability when implanted. For example some studies reported MSC differentiation into osteoblasts after implantation into ventricular tissue (Yoon et al. 2004). This issue needs to be addressed prior full scale therapy.

36.2.1.4 Skeletal Myoblasts

Skeletal myoblasts were among the first cell types considered for cardiac repair. Also called satellite cells, they are found beneath the basal membrane of muscle

tissue and start to proliferate when stimulated by muscle injury or disease (Buckingham and Montarras 2008). Skeletal myoblasts are of special interest for cardiac repair as they can differentiate into nonmuscle cell types (Arsic et al. 2008) and are resistant to ischaemia (Pagani et al. 2003), which is an obvious obstacle to the function of other stem cells in injured myocardium. Animal studies in cardiac disease models have been performed with encouraging results. However, skeletal myoblasts do not fully differentiate into cardiomyocytes in vivo after transplantation. The contracting myotubules do not operate in synchrony with the surrounding myocardium, which is most likely due to a lack of the gap-junction protein connexin 43 activity and lack of electrical coupling with the surrounding cardiac cells (Leobon et al. 2003; Reinecke et al. 2002). First clinical studies were able to proof the feasibility and safety of skeletal myoblast implantation to the heart (Herreros et al. 2003; Menasche et al. 2003; Pagani et al. 2003), yet the seen benefits were only marginal. Moreover these studies raised one considerable concern regarding the use of skeletal myoblasts for cardiac regeneration, which is their potential to create ventricular arrhythmias (Fouts et al. 2006; Itabashi et al. 2005). However more- recent clinical trials did not record an increase of arrhythmic events in vivo after intracardiac injection of skeletal myoblasts (Menasche et al. 2008). Preclinical studies have also shown that induced overexpression of connexin 43 might help to overcome this problem (Abraham et al. 2005).

36.2.1.5 Cardiac Stem Cells

The heart has traditionally been seen as a postmitotic organ, with no further capacity for cardiomyocyte renewal and regeneration. In the last years contradictory data began to accumulate as – under certain pathological conditions, like ischaemia or hypertension – cardiomyocyte proliferation and cell cycling were found to take place in myocardium (Anversa et al. 1990; Beltrami et al. 2001; Bergmann et al. 2009). This idea was challenged when male Y-chromosome positive cardiomyocytes and endothelial cells were found in donor female cardiac tissue after transplantation into a male recipient (Quaini et al. 2004). Furthermore estimates of death rate levels of adult cardiomyocytes led to the consideration of a pool of cardiac progenitor cells (Ellison et al. 2007). Following intensive research discovered several different cell types with stem cell characteristics in the adult heart. These stem cells are described by different patterns. Some cells are dye-negative, as they exclude any vital dyes like Hoechst 33342 or Rhodamin 123. They are also called side populations (SP) cells. Another resident stem cell population is characterized by the expression of c-kit (CD 117). They are located in small clusters in the adult heart. A third population of cells in heart with stem cell features expresses the stem cell antigen 1 (Sca-1). Of these cell types SP cells and Sca-1+ cells are mobilised after cardiac injury (Mouquet et al. 2005; Oh et al. 2003) and Sca-1+ cells have been found to differentiate to cardiomyocytes around the injured area. C-kit+cells have regenerative potential after transplantation, giving rise to cardiomyocytes endothelial cells and smooth muscle cells. C-kit+cell transplantation to injured hearts led to significant improvement in ventricular function (Beltrami et al. 2003). Until today it is unclear,

if these various stem cells are distinct cell types or if they represent different stages of a single cell lineage. It appears that cardiac stem cells reside in specialised niches, which support the growth and maintenance of the cell pool (Fuchs et al. 2004). Possible such niches have been localised all over the myocardium with a concentration in deep tissue at the atria and apex (Beltrami et al. 2003; Urbanek et al. 2005). However these stem cell pools seem to diminish with ageing, a fact that might contribute to the lack of efficacy of regeneration in elderly individuals (Torella et al. 2006). Since it is predominantly the older patients are affected by increased mortality due to cardiomyopathies, attempts to enhance or rejuvenate this senescent stem cell population are of great interest.

There are two new encouraging reports on the therapeutic effects of cardiac stem cells: Autologous CD105+ cardiosphere-derived cells (CDCs), grown in 36 days from endomyocardial biopsy specimens ,significantly reduce scar mass of the infarcted ventricle, when infused intracoronarily 1–3 months after the onset of the infarct (Makkar et al. 2012). There was also an increase of regional contractility, however, there were no relevant changes of ventricular geometry (enddiastolic and endsystolic volume). Anther also promising report showed, that intracoronary infusion of autologous C-kit-positive, lineage-negative cardiac stem cells (CSCs), obtained from atrial tissue during cardiac surgery, increased ejection fraction 4 months thereafter from 30 to 38 % in the majority of treated patients. Moreover, infarct size was considerably reduced (Bolli et al. 2011).

36.2.2 Embryonic Tissue Derived Cells

Embryonic stem cells (ESC), derived form the inner mass of the blastocyst, offer theoretically limitless regenerative capacity, since they are able to give rise to most somatic cell lineages in vivo and in vitro. Furthermore by culturing in various growth media, differentiation can be driven towards a desired cell type such as cardiomyocytes (Odorico et al. 2001). Implantation of these cells into injured cardiac tissue has been tested successfully in preclinical studies (Min et al. 2002). Probably the greatest capacity for cardiac differentiation and long-term survival has been seen in studies using ESCs (van Laake et al. 2008). However there are some major concerns regarding the use of ESC in humans for regenerative purposes. First, the broad differentiation capacity along endo-, ecto- and mesodermal lineages considerably increases the likelihood of teratoma formation (Blum and Benvenisty 2008). Therefore recent experimental results reporting about tumor-resistent, cardiopoietic programming of ESC by modification of host tissue tissue secrete factors are of interest (Behfar et al. 2007). Second, there is increasing evidence that ESC, once thought to be uniquely immunoprivileged, express specific human leukocyte antigen subclasses (Draper et al. 2002). This raises the question how to avoid possible anti-allogenic graft rejection. Immunosuppression with steroids is known to be harmful for ischaemic myocardium(Silverman and Pfeifer 1987). At the moment there is ongoing research to limit the immunogenicity of the cell for allogeneic transplantation.

Finally ESC and their origin have raised a considerable ethical debate. The recent discovery of the possibility to generate ESC-like cells, called inducible pluripotent stem (iPS) cells, by reprogramming adult somatic cells (Aoi et al. 2008; Takahashi et al. 2007), might help to overcome the ethical, immunogeneic and probably tumorogenic problems associated with the use of ESCs.

36.3 Cardiac Cell Therapy, Regenerative Principles

36.3.1 Stem Cell Isolation and Methodological Prerequisites

Important conditions for clinical stem cell therapy are the precise and careful techniques of bone marrow cell preparation, availability of large cell concentrations within the area of interest (border zone of infarction), migration of stem cells into the apoptotic or necrotic myocardial area, and prevention of homing of transplanted cells to other extracardiac organs.

For stem cell transplantation in cardiac diseases, adult bone marrow (80–200 ml) is aspirated under local anaesthesia from the iliac crest. Respective bone marrow stem cell populations then need to be isolated under good manufacturing practice (GMP) conditions. During cell preparation, viability needs to be determined several times and finally must reach around 95 %. All microbiological tests of the clinically used cell preparations must prove negative for endogenous (HIV, HBV, HCV) or exogenous contamination.

36.3.2 Intracoronary Application

One of the most important and crucial methodological questions refers to the optimum mechanism of cell delivery to the heart. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection; assuming normal coronary blood flow of 80 ml/min per 100 g of left ventricular weight, a quantity of 160 ml per left ventricle (assuming a regular left ventricular mass of ~200 g) will flow per minute. This corresponds to only around 3% of cardiac output (assuming a cardiac output of 5,000 ml/min) (Gregg 1963; Strauer 1979). Thus, intravenous application would require many circulation passages to enable infused cells to come in contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Supplying the entire heart muscle compartment by intracoronary administration obviously seems to be advantageous for tissue repair of infarcted heart muscle after interventional reopening of the occluded coronary artery as all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage of the

post-ischemic region (Fuchs et al. 2001; Galinanes et al. 2004). Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched depending on the arterial circulation access of the tissue compartments.

A selective intracoronary delivery route has been developed in interventional cardiology (Strauer et al. 2001, 2002), which minimizes the cell loss due to extraction toward organs of secondary interest by this first pass-like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells are infused by high-pressure injection directly into the necrotic area, and the balloon is kept inflated for 2–4 min; cells are not washed away immediately under these conditions.

Cells are directly transplanted by the intracoronary administration route into the infarcted zone. This is accomplished by a balloon catheter, which is placed within the infarct-related artery. After exact positioning at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) is performed 4 times for 2–4 min each. During this time of vessel occlusion, intracoronary cell transplantation via the balloon catheter is performed, using 4 fractional high-pressure infusions of 5 ml cell suspension, each of which contains 6–8 million mononuclear cells. PTCA thoroughly prevents backflow of cells and at the same time produces a stop-flow beyond the site of balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration is allowed. This migration process is probably only present in injured and ischaemic tissue (Szilvassy et al. 1999). Myocardial ischemia may be the best stimulus for a stem cell to find its optimum myocardial niche (Kamota et al. 2009), probably due to SDF-1 and CXCR4 interrelations (Elmadbouh et al. 2007). Therefore, ischemia-producing stimulus by balloon dilatation during bone marrow cell infusion seems to be necessary for the cells to home into the cardiac niche and for therapeutical effectiveness of cell migration (Sussman 2001; Loffredo et al. 2011). The Intracoronary approach, however, should be reserved for mononuclear bone marrow stem cells, since intracoronary application of cultured cell types like mesenchymal stem cells or skeletal myoblasts could provoke microemboli (Furlani et al. 2009). Due to their size and shape (stellar and spindle-shaped) these cells are more prone to embolisations than bone marrow mononuclear cells. Until now, in all published clinical studies, no case of embolisation by therapeutically used bone marrow cells has been reported.

36.3.3 Endocardial Application

A second interventional delivery route for cardiac stem cell transplantation is the transendocardial injection. In contrast to intracoronary application stem cells can be delivered directly into the target area of the myocardium without dependence on vascular access or sufficient cell migration across the endothelial barrier. Left heart catheterization is performed, followed by subsequent electro mechanical mapping of the left ventricle. In this way areas of viable myocardium (unipolar voltage ≥6.9 mV) within the ischemic region can be identified as specific targets for treatment.

This is followed by stem cell injection into the identified viable myocardium using the NOGA injection catheter, which is placed across the aortic valve into the target area. When in position, periodic cell injection can be performed to the endocardium and myocardium. This interventional approach offers intramyocardial cell delivery similar to the surgical approach with being less invasive at the same time. First clinical studies were able to prove safety and feasibility of the transendocardial route in the setting of chronic ischemic heart disease (Perin et al. 2003) as well as for intractable angina (Losordo et al. 2007). However, orientation by electromechanical mapping is technically demanding and cell loss into the ventricle or wrong injection sites can occur.

36.3.4 Epicardial Application

Surgical stem cell implantation is performed into well exposed ischemic areas, allowing for multiple injections within and principally around the infarct area with a thin needle. First clinical studies performed stem cell injection in combination with coronary artery bypass grafting (CABG). Once the graft-coronary artery anastomoses is completed the ischemic area is visualized and the cells are injected into the border zone of the infarcted area (Stamm et al. 2007). This method has been applied successfully also during off-pump coronary artery bypass grafting as well as stand-alone minimally invasive procedure where cell injection is performed without cardiac arrest. As already described for the transendocardial cell delivery, intramyocardial stem cell injection during surgery seems to overcome the problem linked to insufficient vascularisation, migration and homing transplanted stem cells more likely than the attempts to influence stem cell migration processes in the vasculature and results in a high stem cell persistence in the heart muscle (Kaminski and Steinhoff 2008). However, the results of intramyocardial stem cell injection are difficult to interprete conclusively when performed together with revascularisation procedures like CABG. Therefore recent reports about surgical “stand alone” stem cell therapy are of great interest (Klein et al. 2007; Pompilio et al. 2008). Herein patients improved in myocardial perfusion and clinical symptoms as a result of stem cell injection only via lateral minithoracotomy. Besides distinguishing between stem cell and revascularisation effects on cardiac function this approach could help to further minimize perioperative risks in the context of surgical stem cell therapy (Fig. 36.1).

36.3.5 Cardiac Tissue Engineering

The purpose of cardiac tissue engineering is to replace or repair injured heart muscle effectively. It comprises a biomaterial based ‘vehicle’, either a porous scaffold or dense patch, made of either natural or synthetic polymeric materials, to aid transportation

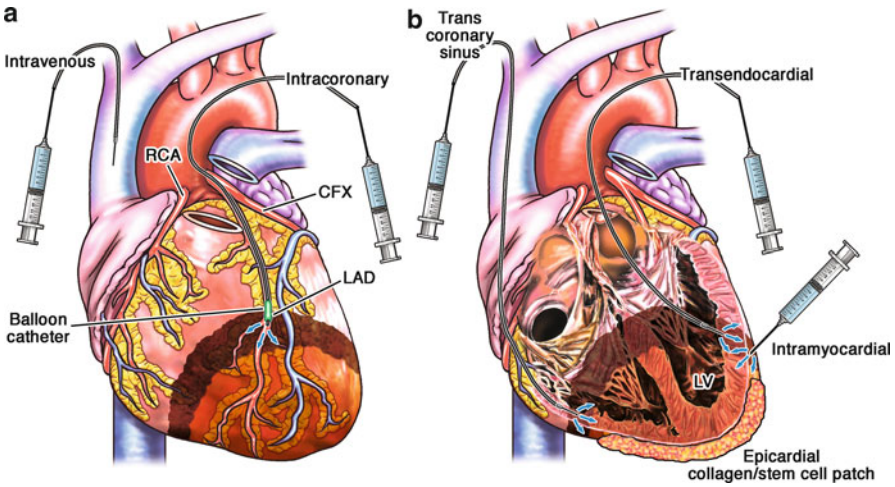


Fig. 36.1 Illustration of intracoronary (interventional) and intramyocardial (surgical) stem cell application. Intracoronary (**a**) and intramyocardial (**b**) transplantation methods in heart disease (Strauer and Steinhoff 2011)

of cells into the diseased region in the heart. Such scaffolds serve several purposes: they allow cell attachment and cell migration, they deliver and retain cells and biochemical factors and they can exert certain mechanical and biological influence to modify the behaviour of the cell phase (Christman and Lee 2006). To achieve sufficient tissue reconstruction, scaffolds have to meet some specific requirements. They should provide high porosity and an adequate pore size to facilitate cell seeding and diffusion throughout the whole structure. Furthermore biodegradability is essential as implanted scaffolds should be absorbed by the surrounding tissue without the need for surgical removal, and the rate of degradation should match the rate of tissue formation as much as possible. This means the scaffold will provide structural integrity as long as the seeded cells produce their own natural matrix structure (Eschenhagen and Zimmermann 2005). The final goal is to allow- by the use of specific biomaterials- the creation of a microatmosphere where exogenous and endogenous cells find the microenvironment optimal for myocardial repair with low scar formation (Chachques 2009). One should also think of implantable, exogenously cellularized matrixes as a supplement of intramyocardial stem cell therapy. This principle has been successfully demonstrated for tissue generated from neonatal cardiomyocytes (Shimizu et al. 2002; Zimmermann et al. 2006). The exogenous matrix could for example help to adjust modified collagen proportions within the scar zone and thereby contribute to the regenerative process (Kutschka et al. 2006). Clinical studies with myocardial tissue engineered constructs have not been performed (Chachques 2009). Preclinical studies on tissue engineered cardiac structures have also focussed on cardiac structures as heart valves and conduction tissue:

Heart valve tissue engineering was started on the basis of seeding biodegradable polymer scaffold in 1995 by the Boston group (Shinoka et al. 1995) showing

successful seeding and implantation in a pulmonary heart valve seeded with autologous vascular cells. This concept was further developed by bioreactor seeding (Hoerstrup et al. 2002) and successfully introduced into cardiac surgery for vena cava vessel conduit replacement in congenital heart surgery by Shinoka (Shinoka and Breuer 2008; Shinoka et al. 2003). The second concept of enzymatic acellularization of biological heart valves was developed by the Hannover group (Steinhoff et al. 2000) and introduced in pediatric heart valve surgery with pulmonary conduit replacement (Cebotari et al. 2006).

Tissue engineering of pacemaker and conduction tissue was only performed experimentally. Transplantation of fetal cardiac cells including conduction tissue into ventricular myocardium was successfully applied in an experimental atrioventricular block model (Ruhparwar et al. 2002). Ex vivo tissue engineered conduction tissue using skeletal myoblasts was successfully applied in a rat model showing function after atrioventricular ablation (Choi et al. 2006). Clinical applications have not been performed with tissue engineered conduction tissue.

36.3.6 Pharmacological Strategies

Pharmacological strategies are aimed to (i) intensify stem cell action by promoting homing into heart muscle and (ii) to stimulate liberation of stem cells from bone marrow. Homing may be enhanced by interventions which increase the oxygen demand of heart muscle thereby inducing relative ischemia (and stimulation of the CXCR4 axis) within the infarct area and its border zone. This may be realised by an increase in contractility (Strauer 1979) and also by provoking coronary steal associated with distribution of blood from diseased to healthy muscle. Intravenous dobutamine during intracoronary stem cell transplantation and up to 24 h thereafter seems is effective. Likewise, dipyridamole (0.5 mg/kg intracoronarily over 10 min) is used immediately prior to stem cell application (Strauer et al. 2008, 2009). Liberation and enhanced production of mononuclear bone marrow cells may be pharmacologically induced by cytokine injection, (e.g. by G-CSF = granulocyte colony stimulating factor) which must be exactly titrated to avoid excess increase in peripheral leucocytes. Clinical studies have not yet confirmed the effectiveness of this kind of therapy (Renault and Losordo 2007; Engelmann et al. 2010). However, recent combination of GM-CSF with SDF-1/CXCR-4 inducing substances as erythropoietin (Klopsch et al. 2009; Brunner et al. 2009), parathyroid hormone (Huber et al. 2010, 2011) and sitagliptin (Theiss et al. 2010, 2011) show promising effects on postinfarction regeneration in experimental models and are introduced in first clinical trials after acute myocardial infarction. First clinical studies employing erythropoietin treatment immediately after interventional reperfusion in acute myocardial infarction show effects on infarction size (Ferrario et al. 2011) and microvascular obstruction (Prunier et al. 2012). Further studies are needed to test clinical safety and efficacy.

36.4 Indications and Clinical Principles

Best examined indications for stem cell therapy are previous myocardial infarction with large infarct area, aneurysm and depressed ejection fraction and heart failure due to chronic myocardial infarction. The age of infarction seems to be irrelevant to regenerative potency of stem cells, since stem cell therapy in old infarcts (older than 8 years) is almost equally effective in comparison to previous infarcts. Further indications are non ischemic cardiomyopathy, diabetic cardiomyopathy, Chagas disease and ischemic mitral regurgitation owing to dysfunction of the left ventricular wall and papillary muscle. The underlying inclusion criteria for cardiac cell therapy are reduction of the ventricular function of cardiac function with an akinetic, viable area which offers no target for standard revascularization procedures. Before cell cardiac transplantation heart failure symptoms, neurohumoral status and the myocardial function and viability should be assessed. Furthermore virus-free test should be performed. Selected patients should be in an early stage of heart failure since stem cell therapy is not an alternative to heart transplantation. The goal of this approach is to avoid or delay organ transplantation (Chachques 2009).

36.4.1 Ischemic Cardiomyopathy

Cardiac stem cell therapy is indicated in patients presenting with impaired left ventricular ejection fraction between 20 and 40 % due to myocardial infarction leading to symptoms of NYHA class II or III with or without angina. The underlying myocardial infarction should be of mild extension-approximately between 9 and 14 cm^2 with the presence of hibernating myocardium in the infarction border zone. Left ventricular wall thickness in echocardiographic evaluation should be greater than 4 mm in order to avoid extramyocardial injection and the risk of iatrogenic ventricular wall injury (Chachques 2009). Early injection after infarction could be beneficial to prevent a large fibrotic scare. On the other hand, since myocardial infarction leads to severe impairment of heart function associated with rhythmic instability and poorer tolerance of additional treatment it might be reasonable to wait for the acute phase to pass until the infarction zone is consolidated. Furthermore cell transplantation should be more effective after the postischemic inflammatory reaction has subsided i.e. after day 8–12 following the acute attack (Chachques 2009; Kaminski and Steinhoff 2008). Stem cell transplantation within the ‘hot’ phase post-myocardial infarction inflammation might lead them to take part in the inflammation cascade rather than in the formation of functional myocardium and vessels (Soeki et al. 2000).

36.4.2 Non Ischemic Cardiomyopathy

Also patients suffering from non ischemic cardiomyopathy could benefit from cardiac stem cell therapy. Preclinical stem cell treatment has been performed successfully in small animal models, such as a canine model of idiopathic dilated

cardiomyopathy and in doxorubicin induced heart failure (Dhein et al. 2006). On the basis of these results clinical trials have been initiated in dilated cardiomyopathy. This first-in-man study of autologous bone marrow cells in dilated cardiomyopathy (Firstin-Man ABCD) investigated 44 patients and the Düsseldorf autologous bone marrow cells in dilated cardiomyopathy trial (Düsseldorf ABCD Trial) investigated 20 patients. In both studies none of the patients had coronary disease (excluded by angiography) or myocarditis (excluded by endomyocardial biopsy). In both trials cell transplantation was performed via the intracoronary administration route in either coronary artery. There has been a significant increase in New York Heart Association Functional Classification in treatment patients in both trials, furthermore ejection fraction improved by 5.4% in the First-in-Man ABCD trial and 8% in the Düsseldorf ABCD Trial. Also first results with paediatric patients suffering from idiopathic dilatative cardiomyopathy showed a clear improvement in left ventricular ejection fraction and left ventricular dimensions after intracoronary stem cell transplantation (Olgunturk et al. 2010). Transplanted cells seem to survive better in the host myocardium since in this pathology myocardial vascularisation is not significantly impaired (Seth et al. 2006). Furthermore data from the First-in-Man ABCD trial suggest that the benefit of stem cell therapy could be a paracrine effect with changes in vascularity (Seth et al. 2006).

36.4.3 Cardiogenic Shock

Only incidental published data are available for cardiogenic shock. A clinical report – in a 64 years old patient with cardiogenic shock after repeated myocardial infarcts – showed marked improvement in ventricular function 4–9-days after intracoronary bone marrow stem cell transplantation (152×10 million) in both coronary arteries (Brehm and Strauer 2007). The ejection fraction increased from 17 to 28% within 9 days after cell therapy, the endsystolic volume decreased from 103 to 09 ml, stroke volume increased from 20 to 35 ml, and 23 days after cell therapy the patient could be transferred to rehabilitation measures.

36.4.4 Diabetic Cardiomyopathy

It is well known that diabetic patients have an increased risk of developing heart failure due to diabetic cardiomyopathy, which is characterized by microvascular pathologies and interstitial fibrosis. Preclinical animal studies have shown that mesenchymal stem cells can, when transplanted, prevent apoptosis of ischemic heart via upregulation of Akt and endothelial nitric-oxide synthase. Furthermore they inhibit myocardial fibrosis of dilated cardiomyopathy by decreasing the expression of matrix metalloproteinase (MMP). Mesenchymal stem cell transplantation significantly increased myocardial arteriolar density in diabetic myocardium leading to an improved cardiac function (Zhang et al. 2008). The capability of mesenchymal stem cells to reduce apoptosis and remodelling processes was further improved after

anoxic preconditioning (Li et al. 2008). Therefore mesenchymal stem cells could be a promising tool to attenuate cardiac remodeling and to improve cardiac function in the setting of diabetic cardiomyopathy.

36.4.5 Chagas Heart Disease

Chagas disease, also called American Trypanosomisasis, is caused by the protozoan Trypanosoma cruzi and remains one of the major health problems in Latin America. In chronic cases 10–30 % of the patients have or will develop cardiomyopathy. In its final stages there are to date no other treatment options than heart transplantation. Four main pathogenetic mechanisms characterize the Chagas heart disease: derangements of the autonomic nervous system, microvascular disturbance, parasite-dependent myocardial aggression and immune mediated myocardial injury (Chachques 2009). On the long run patients develop severe cardiac arrhythmias, dilated cardiomyopathy and heart failure. Since the number of available organs for transplantation is very limited and furthermore late reactivation of the disease after transplantation due to isolated organ lesions is described, cardiac cell therapy is an important option for patients with secondary heart failure caused by Chagas disease. Selective intracoronary infusion of mononuclear stem cells has been performed and resulted in improved cardiac functions and haemodynamics (Soares et al. 2007; Vilas-Boas et al. 2006).

36.4.6 Exclusion Criteria for Cardiac Cell Therapy

Patients presenting with a history of ventricular arrhythmias as well as patients with an implanted cardiac defibrillator (ICD) or candidates for an implantation of an ICD should be evaluated and observed carefully since possibly induced arrhythmias by cell transplantation are a potential complication (Villa et al. 2007), although certain antiarrhythmic properties have to be expected (Dhein et al. 2006). Furthermore patients with hematologic disease should be excluded from bone marrow stem cell transplantation. Also subjects with a history of drug abuse, of cancer, or with active infectious disease (HIV, viral hepatitis) or positive viral test should be excluded (Chachques 2009).

36.4.7 Stem Cells and Arrhythmogenicity of the Heart in Clinical Heart Failure

Stem cell therapy in heart failure is aimed to improve ventricular geometry and function, thereby reducing ventricular arrhythmias by reducing its provoking determinants (increased heart size, wall stress etc.). Studies concerning the rhythmologic effects of different stem cells have to consider that the diseased heart muscle is an

ideal basis for ventricular arrhythmias itself and may alter its electric instability parallel to remodelling. The arrhythmogenic risk in the course of stem cell transplantation depends on the degree of myocardial damage, the specific ability of the stem cell type itself to create arrhythmias and the mode of delivery (and presumably myocardial lesion). Skeletal myoblasts lose its capacity to create connexion 43 over time after transplantation which results in insufficient gap junction formation (Macia and Boyden 2009). Functional integration of skeletal myoblasts therefore is poor and increases arrhythmogenicity. Mesenchymal stem cells have an inhomogenously increased arrhythmogenicity despite good coupling with myocytes., presumably because of increased tissue heterogeneity induced by inexcitable mesenchymal cells (Ly and Nattel 2009). Also cardiac hyperinnervation is discussed.

Bone marrow stem cells in the majority of studies do not provoke arrhythmias. In patients with acute myocardial infarction and in patients with chronic heart failure antiarrhythmic effects have been observed after stem cell therapy (Wollert et al. 2004). However, it is difficult to define a direct antiarrhythmic property. Stem cell therapy leads to ventricular anti-remodelling which itself acts indirectly antiarrhythmic: heart size and wall stress decrease and ejection fraction increases, thus reducing important proarrhythmic factors by virtue of its effects on ventricular geometry and wall dynamics. Moreover, long-term observations following intracoronary stem cell transplantation in acute myocardial infarction demonstrate increased survival of treated patients (Yousef et al. 2009). In advanced cardiac failure due to dilatative cardiomyopathy significant decrease in premature beats and in the occurrence in late potentials were found, which is compatible with a "prorhythmic" effect of bone marrow cells. Moreover, after intracoronary bone marrow cell transplantation, left ventricular synchrony is improved, a result, which may potentially consider BMC therapy suitable for resynchronisation interventions. Stem cells with high potency for transdifferentiation to cardiac myocytes, e.g. embryonic stem cells, seem to possess high arrhythomogenicity, whereas stem cells with low or without transdifferentiation potency, e.g. bone marrow cells, exert "prorhythmic" effects. Independent from the primary arrhythmogenic properties of various types of stem cells, it has to be considered that even in the presence of stem cell induced cardiac excitability the anti-remodelling properties may overcome and improve the arrhythmic problem. In brief, it seems reasonable to assume that the type of stem cell may determine the arrhythmic fate of the heart: bone marrow stem cells seem to be neutral with regard to arrhythmogenity and may even exert antiarrhythmic properties, whereas myoblasts, mesenchymal and preferably embryonic stem cells are prone to inducible arrhythmias.

36.5 Clinical Studies

To date several studies in humans with stem cell transplantation in patients with acute myocardial infarction as well as chronic ischemic heart disease have been performed (Tables 36.1 and 36.2). Although most of these studies have yielded encouraging results the extent to which stem cell transplantation can improve the

Table 36.1 Major clinical trials investigating the treatment of acute myocardial infarction with autologous bone marrow stem cells

Author (year)	Sample size	Primary intervention	Co-intervention mean Stem cell dose (SD)	Time of SC application to AMI	Follow-up duration
Ge et al. (2006)	20	PCI	BMSC aspiration 4×107	Within 7 days	6 months
Huang (2006)	40	PCI	BMSC aspiration 1.8 (4.2)×10^8	Within 7 days	6 months
Janssens et al. (2006)	67	PCI	BMSC aspiration 1.7 (0.72)×108	Within 7 days	4 months
Karpov et al. (2005)	50	PCI	BMSC aspiration 88.5 (49.2)×106	>7 days	6 months
Lunde et al. (2006)	100	PCI	BMSC aspiration 0.68 10×8	Within 7 days	6 months
Wollert et al. (2004)	60	PCI	BMSC aspiration 2.46 (0.94)×109	Within 7 days	6 months
Schachinger et al. (2006)	187	PCI	BMSC aspiration 2.36 (1.74)×108	Within 5 days	4 months
Strauer et al. (2001)	20	PCI	BMSC aspiration 2.8×107	Within 1 day	3 months

SD standard deviation, *SC* stem cells, *PCI* percutaneous coronary intervention, *AMI* acute myocardial infarction, *BMSC* bone marro stem cells

Table 36.2 Major clinical trials investigating the treatment of chronic ischemic heart disease with autologous bone marrow stem cells

Author (year)	Sample size	Primary intervention	Co-intervention; mean stem cell dose (SD)	Route of injection	Follow-up duration
Hendrikx et al. (2006)	20	CABG	BMSC aspiration 60.25 (31)×106	IM	4 months
Mocini et al. (2006)	36	CABG	BMSC aspiration 292 (232)×106	IM	3 months
Perin et al. (2004)	20	NOGA catheterization	BMSC aspiration 25.5 (6.3)×106	IM	12 months
Strauer et al. (2005)	36	PCI	BMSC aspiration 90×106	IC	3 months
Erbs et al. (2005)	26	PCI	BMSC mobilisation (G-CSF) 69(14)106	IC	3 months
Stamm et al. (2007)	40	CABG	BMSC aspiration 5.80×106	IM	6 months
Zhao et al. (2008)	36	CABG	BMSC aspiration 6.59(5.12)108	IM	6 months
Patel et al. (2005)	20	CABG	BMSC aspiration 22×106	IM	6 months

SD standard deviation, *SC* stem cells, *PCI* percutaneous coronary intervention, *CABG* coronary artery bypass grafting, *BMSC* bone marrow stem cells, *IC* intracoronary, *IM* intramyocardial

patients outcome remains unclear since the methods and inclusion criteria used have been quite heterogenous until today. The following chapter will try to give an overview about major interventional and surgical trials carried out so far.

36.5.1 Acute Myocardial Infarction

In acute myocardial infarction, as a domaine of interventional therapy strategies, a variety of studies has demonstrated longstanding (up to 3 years and more) improvement of ventricular performance after using stem cell therapy, resulting in increase in ejection fraction by 4–20 (mean 14%) and decreased infarct size by 3–30% (mean 37%) (Strauer et al. 2001, 2002).

As the time point of stem cell transplantation most studies chose a point between 7 and 8 days after infarction onset. As mentioned already above these data show large variability that not only relates to the biological and specific haemodynamic situation of the infarcted heart, but may depend on different methodological procedures (e.g. the number of transplanted stem cells, mode of balloon-induced preconditioning, time interval between the acute infarct and cell transfer, kind of left ventricular volume determination). However, altogether, there is in all clinical studies unequivocal improvement of performance of the infarcted heart (ejection fraction and/or infarct size) after stem cell therapy of at least 6 %, which is quantitatively more than the sum of the interventional measures (PTCA, stent) and which is achieved in addition to these therapeutic interventions (Assmus et al. 2006; Schachinger et al. 2006). Thus, autologous stem cell therapy represents an innovative and effective procedure for regeneration of impaired heart muscle in the early phase after the infarct (Janssens et al. 2006; Lunde et al. 2005; Wollert et al. 2004)

36.5.2 Chronic Ischemic Heart Disease

To date clinical studies have revealed beneficial stem cell effects in subacute and chronic ischemic heart failure. Several surgical studies performing intramyocardial stem cell transplantation have been designed for this setting. Combined with coronary artery bypass surgery the improvement of cardiac function by the use of bone marrow stem cell has been described as an increase in left ventricular ejection fraction of about 10% (Ahmadi et al. 2007; Stamm et al. 2007; Zhao et al. 2008) and improvement of wall motion caused by enhanced myocardial perfusion. Also studies combining stem cell transplantation with off-pump coronary surgery report similar results (Patel et al. 2005), implicating that cardiac arrest is not a mandatory for safe an efficient stem cell implantation. However these results will always be difficult to interpret conclusively without consideration of revascularisation effects. Therefore recent reports about “stand alone stem cell treatment” for patients with ischemic heart failure are very interesting (Klein et al. 2007). A recent study reported not only

a gain in cardiac function but also a clear improvement in quality of life for patients with chronic ischemic heart disease and refractory angina treated after "stand alone" bone marrow stem cell injection via lateral minithoracotomy (Pompilio et al. 2008).

Interventional studies using intracoronary or endocardial stem cell application have also been performed in the setting of chronic ischemic heart disease. These studies report an improvement of left ventricular ejection fraction in a similar extent like surgical trials. Furthermore, a significant decrease in infarction size and an improved overall myocardial oxygen uptake have been described (Strauer et al. 2005).

Although the results of these trials mentioned above have been promising, there remain open questions. For example if left ventricular ejection fraction alone, as the major outcome parameter so far, characterizes the effects of stem cell treatment adequately. Also several trials performed so far are limited by lack of sham/placebo treatment in control groups. For final evaluation of stem cell related gain in cardiac performance and quality of life, further double blinded Phase III prospective randomised, placebo controlled, clinical outcome trials are needed.

36.5.3 Ongoing Clinical Trials

Several trials running currently try to answer the questions mentioned above. Regarding the effect of intracoronary bone marrow progenitor cell infusion in the setting of acute myocardial infarction placebo controlled Phase III trials like the REGEN-AMI and BAMIare of particular interest. In the field of surgical cell therapy the recently launched PERFECT trial is the first placebo controlled, double blinded, multicenter study investigating the effects of intramyocardial bone marrow stem cell injection combined with CABG surgery. Although representing Phase I and II level the PROMETHEUS study is highly interesting since it represents the first in men study analysing the safety and efficacy of intramyocardial injection of mesenchymal stem cells during CABG in patients scheduled for coronary surgery due to ischemic heart disease, as an alternative cell population to hematopoietic progenitor cell populations mainly used in clinical trials for cardiac regeneration so far. There are several more interesting trials currently recruiting patients (www.clinicaltrials.com) and results from all of these a needed for a valid evaluation of the gain in cardiac function related to stem cell therapy.

36.6 Future Perspectives

Since the first description of the use of bone marrow-derived stem cells for treatment of heart disease in 2001, a large number of clinical studies have been published demonstrating the effectiveness of stem cells in various clinical conditions, but with very different bone marrow preparation techniques. The use of non-standardized cell transplantation procedures is common with large variation in (i) type of transplanted cells, (ii) number of transplanted cells (iii) additive preconditioning measures.

Therefore future studies performed should be aimed to define the optimum technique of cell preparation, to discover the best cell type for myocardial regeneration, to analyse their homing characteristics to the cardiac niche and to other extracardiac organs, to improve cell delivery techniques and to try to establish indications for cell therapy in various heart diseases (Strauer and Kornowski 2003). In realizing these perspectives, joint and cooperative studies between preclinical and clinical research are essential. The mechanisms of stem cell related cardiac repair need to be further investigated and alternative modes of action like paracrine activity and immunomodulation should be considered. The immunomodulatory capacity of mesenchymal stem cells for example could offer new options to supplement the established immunosuppressive therapies in the setting of solid organ transplantation. Furthermore attempts to create dynamic "multi-lineage" cardiac regeneration by combining cell therapy with tissue engineered scaffolds or cardiac resynchronisation therapy (Chachques 2009; Shafy et al. 2009) should be further supported since they offer a realistic perspective to come to an integrated regenerative approach.

References

Abraham MR, Henrikson CA, Tung L, Chang MG, Aon M, Xue T, Li RA, O'Rourke B, Marban E (2005) Antiarrhythmic engineering of skeletal myoblasts for cardiac transplantation. Circ Res 97:159–167

Ahmadi H, Baharvand H, Ashtiani SK, Soleimani M, Sadeghian H, Ardekani JM, Mehrjerdi NZ, Kouhkan A, Namiri M, Madani-Civi M, Fattahi F, Shahverdi A, Dizaji AV (2007) Safety analysis and improved cardiac function following local autologous transplantation of CD133(+) enriched bone marrow cells after myocardial infarction. Curr Neurovasc Res 4:153–160

Alhadlaq A, Mao JJ (2004) Mesenchymal stem cells: isolation and therapeutics. Stem Cells Dev 13:436–448

Allgöwer M (1956) The cellular basis of wound repair. Charles C. Thomas, Springfield

Amado LC, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S, Durand DJ, Fitton T, Kuang JQ, Stewart G, Lehrke S, Baumgartner WW, Martin BJ, Heldman AW, Hare JM (2005) Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl Acad Sci USA 102:11474–11479

Anversa P, Palackal T, Sonnenblick EH, Olivetti G, Capasso JM (1990) Hypertensive cardiomyopathy. Myocyte nuclei hyperplasia in the mammalian rat heart. J Clin Invest 85:994–997

Aoi T, Yae K, Nakagawa M, Ichisaka T, Okita K, Takahashi K, Chiba T, Yamanaka S (2008) Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science (New York, NY) 321:699–702

Aoki J, Serruys PW, van Beusekom H, Ong AT, McFadden EP, Sianos G, van der Giessen WJ, Regar E, de Feyter PJ, Davis HR, Rowland S, Kutryk MJ (2005) Endothelial progenitor cell capture by stents coated with antibody against CD34: the HEALING-FIM (Healthy Endothelial Accelerated Lining Inhibits Neointimal Growth-First In Man) Registry. J Am Coll Cardiol 45:1574–1579

Arsic N, Mamaeva D, Lamb NJ, Fernandez A (2008) Muscle-derived stem cells isolated as nonadherent population give rise to cardiac, skeletal muscle and neural lineages. Exp Cell Res 314:1266–1280

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science (New York, NY) 275:964–967

Assmus B, Honold J, Schachinger V, Britten MB, Fischer-Rasokat U, Lehmann R, Teupe C, Pistorius K, Martin H, Abolmaali ND, Tonn T, Dimmeler S, Zeiher AM (2006) Transcoronary transplantation of progenitor cells after myocardial infarction. N Engl J Med 355:1222–1232

Behfar A, Perez-Terzic C, Faustino RS, Arrell DK, Hodgson DM, Yamada S, Puceat M, Niederlander N, Alekseev AE, Zingman LV, Terzic A (2007) Cardiopoietic programming of embryonic stem cells for tumor-free heart repair. J Exp Med 204:405–420

Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA, Anversa P (2001) Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 344:1750–1757

Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114:763–776

Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J (2009) Evidence for cardiomyocyte renewal in humans. Science (New York, NY) 324:98–102

Blum B, Benvenisty N (2008) The tumorigenicity of human embryonic stem cells. Adv Cancer Res 100:133–158

Bolli R, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, Beache GM, Wagner SG, Leri A, Hosoda T, Sanada F, Elmore JB, Goichberg P, Cappetta D, Solankhi NK, Fahsah I, Rokosh DG, Slaughter MS, Kajstura J, Anversa P (2011) Cardiac stem cells in patients with ischaemic cardio myopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet 378(9806):1847–1857, Epub 2011 Nov 14

Brehm M, Strauer BE (2007) Reversal of therapy-resistant cardiogenic shock after intracoronary transplantation of adult autologous bone marrow derived stem cells. Dtsch Med Wochenschr 132:1944–1948

Brunner S, Winogradow J, Huber BC, Zaruba MM, Fischer R, David R, Assmann G, Herbach N, Wanke R, Mueller-Hoecker J, Franz WM (2009) Erythropoeitin administration after myocardial infarction in mice attenuates ischemic cardiobymopathy associated with enhanced homing of bone marrow-derived progenitor cells bia the CXCR-4/SDF-1 axis. FASEB J 23(2):351–361, Epub 2008 Sep 30

Buckingham M, Montarras D (2008) Skeletal muscle stem cells. Curr Opin Genet Dev 18:330–336

Cebotari S, Lichtenberg A, Tudorache I, Hilfiker A, Mertsching H, Leyh R, Breymann T, Kallenbach K, Maniuc L, Batrinac A, Repin O, Maliga O, Ciubotaru A, Haverich A (2006) Clinical application of tissue engineered human heart valves using autologous progenitor cells. Circulation 114:I132–I137

Chachques JC (2009) Cellular cardiac regenerative therapy in which patients? Expert Rev Cardiovasc Ther 7:911–919

Choi YH, Stamm C, Hammer PE, Kwaku KF, Marler JJ, Friehs I, Jones M, Rader CM, Roy N, Eddy MT, Triedman JK, Walsh EP, McGowan FX Jr, del Nido PJ, Cowan DB (2006) Cardiac conduction through engineered tissue. Am J Pathol 169:72–85

Christman KL, Lee RJ (2006) Biomaterials for the treatment of myocardial infarction. J Am Coll Cardiol 48:907–913

Dai W, Hale SL, Martin BJ, Kuang JQ, Dow JS, Wold LE, Kloner RA (2005) Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short- and long-term effects. Circulation 112:214–223

Dhein S, Garbade J, Rouabah D, Abraham G, Ungemach FR, Schneider K, Ullmann C, Aupperle H, Gummert JF, Mohr FW (2006) Effects of autologous bone marrow stem cell transplantation on beta-adrenoceptor density and electrical activation pattern in a rabbit model of non-ischemic heart failure. J Cardiothorac Surg 1:17

Draper JS, Pigott C, Thomson JA, Andrews PW (2002) Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J Anat 200:249–258

Ellison GM, Torella D, Karakikes I, Nadal-Ginard B (2007) Myocyte death and renewal: modern concepts of cardiac cellular homeostasis. Nat Clin Pract 4(Suppl 1):S52–S59

Elmadbouh I, Haider H, Jiang S, Idris NM, Lu G, Ashraf M (2007) Ex vivo delivered stromal cell-derived factor-1alpha promotes stem cell homing and induces angiomyogenesis in the infarcted myocardium. J Mol Cell Cardiol 42:792–803

Engelmann MG, Theiss HD, Theiss C, Henschel V, Huber A, Wintersperger BJ, Schoenberg SO, Steinbeck G, Franz WM (2010) G-CSF in patients suffering from late revascularized ST-elevation myocardial infarction: final 1-year-results of the G-CSF-STEMI Trial. Int J Cardiol 144(3):399–404, Epub 2009 May 27

Erbs S, Linke A, Adams V, Lenk K, Thiele H, Diederich KW, Emmrich F, Kluge R, Kendziorra K, Sabri O, Schuler G, Hambrecht R (2005) Transplantation of blood-derived progenitor cells after recanalization of chronic coronary artery occlusion: first randomized and placebo-controlled study. Circ Res 97(8):756–762

Eschenhagen T, Zimmermann WH (2005) Engineering myocardial tissue. Circ Res 97:1220–1231

Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. Science (New York, NY) 279:1528–1530

Ferrario M, Arbustini E, Massa M, Rosti V, Marziliano N, Raineri C, Campanelli R, Bertoletti A, De Ferrari GM, Klersy C, Angoli L, Bramucci E, Marinoni B, Ferlini M, Moretti E, Raisaro A, Repetto A, Schwartz PJ, Tavazzi L (2011) High-dose erythropoeitin in patients with acute myocardial infarction: a pilot, randomised placebo-controlled study. Int J Cardiol 147(1):124–131, Epub 2009 Nov 10

Fouts K, Fernandes B, Mal N, Liu J, Laurita KR (2006) Electrophysiological consequence of skeletal myoblast transplantation in normal and infarcted canine myocardium. Heart Rhythm 3:452–461

Fuchs S, Baffour R, Zhou YF, Shou M, Pierre A, Tio FO, Weissman NJ, Leon MB, Epstein SE, Kornowski R (2001) Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. J Am Coll Cardiol 37:1726–1732

Fuchs E, Tumbar T, Guasch G (2004) Socializing with the neighbours: stem cells and their niche. Cell 116:769–778

Furlani D, Ugurlucan M, Ong L, Bieback K, Pittermann E, Westien I, Wang W, Yerebakan C, Li W, Gaebel R, Li RK, Vollmar B, Steinhoff G, Ma N (2009) Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal stem cells and intravital microscopy. Microvasc Res 77:370–376

Galinanes M, Loubani M, Davies J, Chin D, Pasi J, Bell PR (2004) Autotransplantation of unmanipulated bone marrow into scarred myocardium is safe and enhances cardiac function in humans. Cell Transplant 13:7–13

Ge J, Li Y, Qian J, Shi J, Wang Q, Niu Y, Fan B, Liu X, Zhang S, Sun A, Zou Y (2006) Efficacy of emergent transcatheter transplantation of stem cells for treatment of acute myocardial infarction (TCT-STAMI). Heart 92(12):1764–1767

Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, Rafii S (2001) Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res 88:167–174

Goodell MA, Jackson KA, Majka SM, Mi T, Wang H, Pocius J, Hartley CJ, Majesky MW, Entman ML, Michael LH, Hirschi KK (2001) Stem cell plasticity in muscle and bone marrow. Ann N Y Acad Sci 938:208–218; discussion 218–220

Gregg F (1963) Blodd supply to the heart. In: Hamilton WF, Dow P (eds) Handbook of physiology. American Physiological Society, Washington DC, pp 1517–1584

Hendrikx M, Hensen K, Clijsters C, Jongen H, Koninckx R, Bijnens E, Ingels M, Jacobs A, Geukens R, Dendale P, Vijgen J, Dilling D, Steels P, Mees U, Rummens JL (2006) Recovery of regional but not global contractile function by the direct intramyocardial autologous bone marrow transplantation: results from a randomized controlled clinical trial. Circulation 114(1 Suppl):I101–I107

Herreros J, Prosper F, Perez A, Gavira JJ, Garcia-Velloso MJ, Barba J, Sanchez PL, Canizo C, Rabago G, Marti-Climent JM, Hernandez M, Lopez-Holgado N, Gonzalez-Santos JM, Martin-

Luengo C, Alegria E (2003) Autologous intramyocardial injection of cultured skeletal muscle-derived stem cells in patients with non-acute myocardial infarction. Eur Heart J 24:2012–2020

Hoerstrup SP, Kadner A, Melnitchouk S, Trojan A, Eid K, Tracy J, Sodian R, Visjager JF, Kolb SA, Grunenfelder J, Zund G, Turina MI (2002) Tissue engineering of functional trileaflet heart valves from human marrow stromal cells. Circulation 106:I143–I150

Huber BC, Fischer R, Brunner S, Groebner M, Rischpler C, Segeth A, Zaruba MM, Wollenweber T, Hacker M, Franz WM (2010) Comparison of parathyroid hormone and G-CSF treatment after myocardial infarction on perfusion and stem cell homing. Am J Physiol Heart Circ Physiol 298(5):H1466–H1471, Epub 2010 Mar 5

Huber BC, Brunner S, Segeth A, Nathan P, Fischer R, Zaruba MM, Vallaster M, Theiss HD, David R, Gerbitz A, Franz WM (2011) Parathyroid hormone is a DPP-IV inhibitor and increases SDF-1-driven homing of CXCR4(+) stem cells into the ischaemic heart. Cardiovasc Res 90(3):529–537, Epub 2011 Jan 18

Itabashi Y, Miyoshi S, Yuasa S, Fujita J, Shimizu T, Okano T, Fukuda K, Ogawa S (2005) Analysis of the electrophysiological properties and arrhythmias in directly contacted skeletal and cardiac muscle cell sheets. Cardiovasc Res 67:561–570

Iwasaki H, Kawamoto A, Ishikawa M, Oyamada A, Nakamori S, Nishimura H, Sadamoto K, Horii M, Matsumoto T, Murasawa S, Shibata T, Suehiro S, Asahara T (2006) Dose-dependent contribution of CD34-positive cell transplantation to concurrent vasculogenesis and cardiomyogenesis for functional regenerative recovery after myocardial infarction. Circulation 113:1311–1325

Janssens S, Dubois C, Bogaert J, Theunissen K, Deroose C, Desmet W, Kalantzi M, Herbots L, Sinnaeve P, Dens J, Maertens J, Rademakers F, Dymarkowski S, Gheysens O, Van Cleemput J, Bormans G, Nuyts J, Belmans A, Mortelmans L, Boogaerts M, Van de Werf F (2006) Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. Lancet 367:113–121

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49

Joggerst SJ, Hatzopoulos AK (2009) Stem cell therapy for cardiac repair: benefits and barriers. Expert Rev Mol Med 11:e20

Jujo K, Ii M, Losordo DW (2008) Endothelial progenitor cells in neovascularization of infarcted myocardium. J Mol Cell Cardiol 45:530–544

Kaminski A, Steinhoff G (2008) Current status of intramyocardial bone marrow stem cell transplantation. Semin Thorac Cardiovasc Surg 20:119–125

Kamota T, Li TS, Morikage N, Murakami M, Ohshima M, Kubo M, Kobayashi T, Mikamo A, Ikeda Y, Matsuzaki M, Hamano K (2009) Ischemic pre-conditioning enhances the mobilization and recruitment of bone marrow stem cells to protect against ischemia/reperfusion injury in the late phase. J Am Coll Cardiol 53:1814–1822

Karpov RS, Popov SV, Markov VA, Suslova TE, Ryabov VV, Poponina YS, Krylov AL, Sazonova SV (2005) Autologous mononuclear bone marrow cells during reparative regeneratrion after acute myocardial infarction. Bull Exp Biol Med 140:640–643

Klein HM, Ghodsizad A, Marktanner R, Poll L, Voelkel T, Mohammad Hasani MR, Piechaczek C, Feifel N, Stockschlaeder M, Burchardt ER, Kar BJ, Gregoric I, Gams E (2007) Intramyocardial implantation of CD133+ stem cells improved cardiac function without bypass surgery. Heart Surg Forum 10:E66–E69

Klopsch C, Furlani D, Gäbel R, Li W, Pittermann E, Ugurlucan M, Kundt G, Zingler C, Titze U, Wang W, Ong LL, Wagner K, Li RK, Ma N, Steinhoff G (2009) Intracardiac injection of erythropoeitin induces stem cell recruitment and improves cardiac functions in a rat myocardial infarction model. J Cell Mol Med 13(4):664–679

Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S (2001) Neovascularization of ischemic myocardium by human bone-marrow-derived

angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 7:430–436
Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105:369–377
Kutschka I, Chen IY, Kofidis T, Arai T, von Degenfeld G, Sheikh AY, Hendry SL, Pearl J, Hoyt G, Sista R, Yang PC, Blau HM, Gambhir SS, Robbins RC (2006) Collagen matrices enhance survival of transplanted cardiomyoblasts and contribute to functional improvement of ischemic rat hearts. Circulation 114:I167–I173
Leobon B, Garcin I, Menasche P, Vilquin JT, Audinat E, Charpak S (2003) Myoblasts transplanted into rat infarcted myocardium are functionally isolated from their host. Proc Natl Acad Sci USA 100:7808–7811
Li JH, Zhang N, Wang JA (2008) Improved anti-apoptotic and anti-remodeling potency of bone marrow mesenchymal stem cells by anoxic pre-conditioning in diabetic cardiomyopathy. J Endocrinol Invest 31:103–110
Loffredo FS, Steinhauser ML, Gannon J, Lee RT (2011) Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. Cell Stem Cell 8:389–398
Losordo DW, Schatz RA, White CJ, Udelson JE, Veereshwarayya V, Durgin M, Poh KK, Weinstein R, Kearney M, Chaudhry M, Burg A, Eaton L, Heyd L, Thorne T, Shturman L, Hoffmeister P, Story K, Zak V, Dowling D, Traverse JH, Olson RE, Flanagan J, Sodano D, Murayama T, Kawamoto A, Kusano KF, Wollins J, Welt F, Shah P, Soukas P, Asahara T, Henry TD (2007) Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina: a phase I/IIa double-blind, randomized controlled trial. Circulation 115:3165–3172
Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Forfang K (2005) Autologous stem cell transplantation in acute myocardial infarction: the ASTAMI randomized controlled trial. Intracoronary transplantation of autologous mononuclear bone marrow cells, study design and safety aspects. Scand Cardiovasc J 39:150–158
Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Egeland T, Endresen K, Ilebekk A, Mangschau A, Fjeld JG, Smith HJ, Taraldsrud E, Grøgaard HK, Bjørnerheim R, Brekke M, Müller C, Hopp E, Ragnarsson A, Brinchmann JE, Forfang K (2006) Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. N Engl J Med 355(12):1199–1209
Ly HQ, Nattel S (2009) Stem cells are not proarrhythmic: letting the genie out of the bottle. Circulation 119:1824–1831
Ma N, Ladilov Y, Kaminski A, Piechaczek C, Choi YH, Li W, Steinhoff G, Stamm C (2006) Umbilical cord blood cell transplantation for myocardial regeneration. Transplant Proc 38:771–773
Macia E, Boyden PA (2009) Stem cell therapy is proarrhythmic. Circulation 119:1814–1823
Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S (1999) Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103:697–705
Makkar RR, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, Czer LS, Marbán L, Mendizabal A, Johnston PV, Russell SD, Schuleri KH, Lardo AC, Gerstenblith G, Marbán E (2012) Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet 379(9819):895–904
Menasche P, Hagege AA, Scorsin M, Pouzet B, Desnos M, Duboc D, Schwartz K, Vilquin JT, Marolleau JP (2001) Myoblast transplantation for heart failure. Lancet 357:279–280
Menasche P, Hagege AA, Vilquin JT, Desnos M, Abergel E, Pouzet B, Bel A, Sarateanu S, Scorsin M, Schwartz K, Bruneval P, Benbunan M, Marolleau JP, Duboc D (2003) Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. J Am Coll Cardiol 41:1078–1083
Menasche P, Alfieri O, Janssens S, McKenna W, Reichenspurner H, Trinquart L, Vilquin JT, Marolleau JP, Seymour B, Larghero J, Lake S, Chatellier G, Solomon S, Desnos M, Hagege

AA (2008) The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. Circulation 117: 1189–1200

Min JY, Yang Y, Converso KL, Liu L, Huang Q, Morgan JP, Xiao YF (2002) Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. J Appl Physiol 92:288–296

Mocini D, Staibano M, Mele L, Giannantoni P, Menichella G, Colivicchi F, Sordini P, Salera P, Tubaro M, Santini M (2006) Autologous bone marrow mononuclear cell transplantation in patients undergoing coronary artery bypass grafting. Am J Heart 151(1):192–197

Mouquet F, Pfister O, Jain M, Oikonomopoulos A, Ngoy S, Summer R, Fine A, Liao R (2005) Restoration of cardiac progenitor cells after myocardial infarction by self-proliferation and selective homing of bone marrow-derived stem cells. Circ Res 97:1090–1092

Nygren JM, Jovinge S, Breitbach M, Sawen P, Roll W, Hescheler J, Taneera J, Fleischmann BK, Jacobsen SE (2004) Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med 10:494–501

Odorico JS, Kaufman DS, Thomson JA (2001) Multilineage differentiation from human embryonic stem cell lines. Stem Cells (Dayton, Ohio) 19:193–204

Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML, Schneider MD (2003) Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci USA 100:12313–12318

Olgunturk R, Kula S, Sucak GT, Ozdogan ME, Erer D, Saygili A (2010) Peripheric stem cell transplantation in children with dilated cardiomyopathy: preliminary report of first two cases. Pediatr Transplant 14(2):257–260

Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410(6829):701–705

Pagani FD, DerSimonian H, Zawadzka A, Wetzel K, Edge AS, Jacoby DB, Dinsmore JH, Wright S, Aretz TH, Eisen HJ, Aaronson KD (2003) Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation. J Am Coll Cardiol 41:879–888

Patel AN, Geffner L, Vina RF, Saslavsky J, Urschel HC Jr, Kormos R, Benetti F (2005) Surgical treatment for congestive heart failure with autologous adult stem cell transplantation: a prospective randomized study. J Thorac Cardiovasc Surg 130:1631–1638

Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Mesquita CT, Rossi MI, Carvalho AC, Dutra HS, Dohmann HJ, Silva GV, Belem L, Vivacqua R, Rangel FO, Esporcatte R, Geng YJ, Vaughn WK, Assad JA, Mesquita ET, Willerson JT (2003) Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation 107:2294–2302

Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Silva GV, Mesquita CT, Belém L, Vaughn WK, Rangel FO, Assad JA, Carvalho AC, Branco RV, Rossi MI, Dohmann HJ, Willerson JT (2004) Improved exercise capacity and ischemia 6 and 12 months after transendocardial injection of autologous bone marrow mononuclear cells for ischemic cardiomyopathy. Circulation 110(11 Suppl 1):II213–II218

Pfeffer MA, Braunwald E (1990) Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation 81:1161–1172

Pompilio G, Steinhoff G, Liebold A, Pesce M, Alamanni F, Capogrossi MC, Biglioli P (2008) Direct minimally invasive intramyocardial injection of bone marrow-derived AC133+ stem cells in patients with refractory ischemia: preliminary results. Thorac Cardiovasc Surg 56:71–76

Prunier F, Bière L, Gilard M, Boschat J, Mouquet F, Bauchart JJ, Charbonnier B, Genée O, Guérin P, Warin-Fresse K, Durand E, Lafont A, Christiaens L, Abi-Khalil W, Delépine S, Benard T, Furber A (2012) Single high-dose erythropoeitin administration immediately after reperfusion in patients with ST-segment elevation myocardial infarction: results of the erythropoietin in myocardial infarction trial. Am Heart J 163(2):200–7.e1

Quaini F, Urbanek K, Graiani G, Lagrasta C, Maestri R, Monica M, Boni A, Ferraro F, Delsignore R, Tasca G, Leri A, Kajstura J, Quaini E, Anversa P (2004) The regenerative potential of the human heart. Int J Cardiol 95(Suppl 1):S26–S28

Reinecke H, Poppa V, Murry CE (2002) Skeletal muscle stem cells do not transdifferentiate into cardiomyocytes after cardiac grafting. J Mol Cell Cardiol 34:241–249

Ren G, Michael LH, Entman ML, Frangogiannis NG (2002) Morphological characteristics of the microvasculature in healing myocardial infarcts. J Histochem Cytochem 50:71–79

Renault MA, Losordo DW (2007) Therapeutic myocardial angiogenesis. Microvasc Res 74:159–171

Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM (2001) Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 98:2615–2625

Ruhparwar A, Tebbenjohanns J, Niehaus M, Mengel M, Irtel T, Kofidis T, Pichlmaier AM, Haverich A (2002) Transplanted fetal cardiomyocytes as cardiac pacemaker. Eur J Cardiothorac Surg 21:853–857

Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, Yu J, Corti R, Mathey DG, Hamm CW, Suselbeck T, Assmus B, Tonn T, Dimmeler S, Zeiher AM (2006) Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 355:1210–1221

Seth S, Narang R, Bhargava B, Ray R, Mohanty S, Gulati G, Kumar L, Reddy KS, Venugopal P (2006) Percutaneous intracoronary cellular cardiomyoplasty for nonischemic cardiomyopathy: clinical and histopathological results: the first-in-man ABCD (Autologous Bone Marrow Cells in Dilated Cardiomyopathy) trial. J Am Coll Cardiol 48:2350–2351

Shafy A, Lavergne T, Latremouille C, Cortes-Morichetti M, Carpentier A, Chachques JC (2009) Association of electrostimulation with cell transplantation in ischemic heart disease. J Thorac Cardiovasc Surg 138:994–1001

Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MA, Storb RF, Hammond WP (1998) Evidence for circulating bone marrow-derived endothelial cells. Blood 92:362–367

Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M, Okano T (2002) Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res 90:e40

Shinoka T, Breuer C (2008) Tissue-engineered blood vessels in pediatric cardiac surgery. Yale J Biol Med 81:161–166

Shinoka T, Breuer CK, Tanel RE, Zund G, Miura T, Ma PX, Langer R, Vacanti JP, Mayer JE Jr (1995) Tissue engineering heart valves: valve leaflet replacement study in a lamb model. Ann Thorac Surg 60:S513–S516

Shinoka T, Matsumura K, Hibino N, Naito Y, Murata A, Kosaka Y, Kurosawa H (2003) Clinical practice of transplantation of regenerated blood vessels using bone marrow cells. Nihon Naika Gakkai zasshi 92:1776–1780

Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y, Imaizumi T (2001) Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation 103:2776–2779

Silverman HS, Pfeifer MP (1987) Relation between use of anti-inflammatory agents and left ventricular free wall rupture during acute myocardial infarction. Am J Cardiol 59:363–364

Soares MB, Garcia S, Campos de Carvalho AC, Ribeiro dos Santos R (2007) Cellular therapy in Chagas' disease: potential applications in patients with chronic cardiomyopathy. Regen Med 2:257–264

Soeki T, Tamura Y, Shinohara H, Tanaka H, Bando K, Fukuda N (2000) Serial changes in serum VEGF and HGF in patients with acute myocardial infarction. Cardiology 93:168–174

Stamm C, Kleine HD, Choi YH, Dunkelmann S, Lauffs JA, Lorenzen B, David A, Liebold A, Nienaber C, Zurakowski D, Freund M, Steinhoff G (2007) Intramyocardial delivery of CD133+ bone marrow cells and coronary artery bypass grafting for chronic ischemic heart disease: safety and efficacy studies. J Thorac Cardiovasc Surg 133:717–725

Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schumichen C, Nienaber CA, Freund M, Steinhoff G (2003) Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 361:45–46

Steinhoff G, Stock U, Karim N, Mertsching H, Timke A, Meliss RR, Pethig K, Haverich A, Bader A (2000) Tissue engineering of pulmonary heart valves on allogenic acellular matrix conduits: in vivo restoration of valve tissue. Circulation 102:III50–III55

Strauer BE (1979) Myocardial oxygen consumption in chronic heart disease: role of wall stress, hypertrophy and coronary reserve. Am J Cardiol 44:730–740

Strauer BE, Kornowski R (2003) Stem cell therapy in perspective. Circulation 107:929–934

Strauer BE, Steinhoff G (2011) 10 years of intracoronary and intramyocardial bone marrow stem cell therapy of the heart. J Am Coll Cardiol 58:1095–1104

Strauer BE, Brehm M, Zeus T, Gattermann N, Hernandez A, Sorg RV, Kogler G, Wernet P (2001) Intracoronary, human autologous stem cell transplantation for myocardial regeneration following myocardial infarction. Dtsch Med Wochenschr 126:932–938

Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, Kogler G, Wernet P (2002) Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 106:1913–1918

Strauer BE, Brehm M, Zeus T, Bartsch T, Schannwell C, Antke C, Sorg RV, Kogler G, Wernet P, Muller HW, Kostering M (2005) Regeneration of human infarcted heart muscle by intracoronary autologous bone marrow cell transplantation in chronic coronary artery disease: the IACT Study. J Am Coll Cardiol 46:1651–1658

Strauer BE, Brehm M, Schannwell CM (2008) The therapeutic potential of stem cells in heart disease. Cell Prolif 41(Suppl 1):126–145

Strauer BE, Ott G, Schannwell CM, Brehm M (2009) Bone marrow cells to improve ventricular function. Heart (British Cardiac Society) 95:98–99

Sussman M (2001) Cardiovascular biology. Hearts and bones. Nature 410:640–641

Szilvassy SJ, Bass MJ, Van Zant G, Grimes B (1999) Organ-selective homing defines engraftment kinetics of murine hematopoietic stem cells and is compromised by ex vivo expansion. Blood 93:1557–1566

Takahashi K, Okita K, Nakagawa M, Yamanaka S (2007) Induction of pluripotent stem cells from fibroblast cultures. Nat Protoc 2:3081–3089

Theiss HD, Brenner C, Engelmann MG, Zaruba MM, Huber B, Henschel V, Mansmann U, Wintersperger B, Reiser M, Steinbeck G, Franz WM (2010) Safety and efficacy of SITAgliptin plus GRanulocyte-colony-stimulating factor in patients suffering from Acute Myocardial Infarction (SITAGRAMI-Trial)-rationale, design and first interim analysis. Int J Cardiol 145(2):282–284, Epub 2010 Jan 3

Theiss HD, Vallaster M, Rischpler C, Krieg L, Zaruba MM, Brunner S, Vanchev Y, Fischer R, Gröbner M, Huber B, Wollenweber T, Assmann G, Mueller-Hoecker J, Hacker M, Franz WM (2011) Dual stem cell therapy after myocardial infarction acts specifically by enhanced homing via the SDF-1/CXCR4 axis. Stem Cell Res 7(3):244–255, Epub 2011 May 16

Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD (2002) Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 105:93–98

Torella D, Ellison GM, Mendez-Ferrer S, Ibanez B, Nadal-Ginard B (2006) Resident human cardiac stem cells: role in cardiac cellular homeostasis and potential for myocardial regeneration. Nat Clin Pract 3(Suppl 1):S8–S13

Tse HF, Yiu KH, Lau CP (2007) Bone marrow stem cell therapy for myocardial angiogenesis. Curr Vasc Pharmacol 5:103–112

Urbanek K, Torella D, Sheikh F, De Angelis A, Nurzynska D, Silvestri F, Beltrami CA, Bussani R, Beltrami AP, Quaini F, Bolli R, Leri A, Kajstura J, Anversa P (2005) Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci USA 102:8692–8697

van Laake LW, Passier R, Doevendans PA, Mummery CL (2008) Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents. Circ Res 102:1008–1010

Vilas-Boas F, Feitosa GS, Soares MB, Mota A, Pinho-Filho JA, Almeida AJ, Andrade MV, Carvalho HG, Dourado-Oliveira A, Ribeiro-dos-Santos R (2006) Early results of bone marrow cell transplantation to the myocardium of patients with heart failure due to Chagas disease. Arq Bras Cardiol 87:159–166

Villa A, Sanchez PL, Fernandez-Aviles F (2007) Ventricular arrhythmias following intracoronary bone marrow stem cell transplantation. Europace 9:1222–1223

Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein B, Ganser A, Drexler H (2004) Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. Lancet 364:141–148

Yoon YS, Park JS, Tkebuchava T, Luedeman C, Losordo DW (2004) Unexpected severe calcification after transplantation of bone marrow cells in acute myocardial infarction. Circulation 109:3154–3157

Yousef M, Schannwell CM, Kostering M, Zeus T, Brehm M, Strauer BE (2009) The BALANCE Study: clinical benefit and long-term outcome after intracoronary autologous bone marrow cell transplantation in patients with acute myocardial infarction. J Am Coll Cardiol 53:2262–2269

Zhang N, Li J, Luo R, Jiang J, Wang JA (2008) Bone marrow mesenchymal stem cells induce angiogenesis and attenuate the remodeling of diabetic cardiomyopathy. Exp Clin Endocrinol Diabetes 116:104–111

Zhao Q, Sun Y, Xia L, Chen A, Wang Z (2008) Randomized study of mononuclear bone marrow cell transplantation in patients with coronary surgery. Ann Thorac Surg 86:1833–1840

Zimmermann WH, Melnychenko I, Wasmeier G, Didie M, Naito H, Nixdorff U, Hess A, Budinsky L, Brune K, Michaelis B, Dhein S, Schwoerer A, Ehmke H, Eschenhagen T (2006) Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. Nat Med 12:452–458

Chapter 37
Liver

Amar Deep Sharma, Razvan Iacob, Tobias Cantz, Michael P. Manns, and Michael Ott

Abstract The liver has adapted to the inflow of ingested toxins by the evolutionary development of unique self-regenerative properties and responds to injury or tissue loss by rapid restoration of the original organ mass. This high regenerative capacity is sufficient to restore normal volume and function in most forms of acute liver injury and medical interventions are not required. Regenerative therapies in hepatology rather aim to enhance repair mechanisms of the liver in situations, where the capacity to regenerate is severely impaired. Alternatively, regenerative technologies are applied to solve so far unmet medical needs. The development of such therapies requires a fundamental understanding of the (patho-) physiology of the liver. In this chapter we discuss the emerging medical approaches for acute liver failure, chronic liver failure and hereditary liver diseases, which are based on technologies such as (stem) cell therapy, tissue engineering, bio-artificial devices or gene therapies. Translation from the laboratory bench into routine clinical applications will also

A.D. Sharma, Ph.D. • T. Cantz, M.D.
Junior Research Group "Stem Cell Biology", Cluster of Excellence REBIRTH,
Hannover Medical School , Carl-Neuberg-Str. 1, 30623 Hannover, Germany

R. Iacob, M.D.
University of Medicine and Pharmacy "Carol Davila",
Str. Traian Vuia, no. 6, Sector 2, Bucharest 020956, Romania

M.P. Manns, M.D.
Department of Gastroenterology, Hepatology and Endocrinology, Centre for Internal Medicine,
Hannover Medical School (MHH), Carl-Neuberg-Str. 1, 30625 Hannover, Germany

M. Ott, M.D. (✉)
Department of Gastroenterology, Hepatology and Endocrinology,
Hannover Medical School, Hannover, Germany

TWINCORE, Centre for Experimental and Clinical Infection Research,
Feodor-Lynen-Str. 7, 30625 Hannover, Germany
e-mail: OTT-MHH@gmx.de

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_37,

require consideration of the legal framework for "advanced therapy medicinal products" (ATMP) as well as "state of the art" manufacturing (GMP) and good clinical practice (GCP) guidelines.

37.1 Physiology and Pathophysiology of Liver Regeneration

The liver, the largest internal organ of the body, comprises about 1/50th of the total adult body weight (Sherlock and Dooley 2002), receives approximately 25% of cardiac output (Schiff et al. 2007) and consists of an exceptional anatomical structure in both biliary system and vasculature. The biliary system, an exocrine system in the liver, connects the apical surface of every single hepatocyte to the duodenum through bile cannaliculi, which drain into the canals of Hering and finally into bile ducts (Burt et al. 2007). The terminal branches of the hepatic portal vein and hepatic artery enter the liver sinusoids, which are characterised by fenestrated and discontinuous endothelium (Sherlock and Dooley 2002; McCuskey 1994). No basement membrane lines the sinusoid, which allows higher permeability and direct transfer of particles less than 100 nm from the vessels to the basolateral surface of the hepatocytes.

In the absence of injury the adult liver is a quiescent organ and as few as one out of 3,000 hepatocytes divides at a given time point to maintain the physiological liver mass. In situations of acute liver damage or surgical loss of liver mass, however, cell proliferation is extensively stimulated until the tissue mass has been restored (Fausto et al. 2006). In 1 week up to 75% surgically removed liver mass can be regenerated in rodents (Michalopoulos and DeFrances 1997). The parenchymal regeneration after necrogenic or surgical loss of liver tissue originates from extensive proliferation of the mature parenchymal liver cells (hepatocytes and cholangiocytes). In a young adult rat or mouse, approximately 95% of hepatocytes enter cell cycle during the first 3 days after extensive hepatectomy. Although the term "liver regeneration" is commonly used, restoration of the liver mass after partial hepatectomy is actually a form of compensatory growth of the remaining liver.

In the regenerative phase after acute liver injury or tissue loss the liver immediately induces more than 100 genes, which are not expressed in normal liver (Taub 1996, 2004). The functions served are several and many of these genes appear to play an essential role, however, the precise role of the many genes expressed early in liver regeneration is often not yet clear. The early changes in gene expression reflect both the entry of hepatocytes into the cell cycle as well as the orchestration of specific adjustments that hepatocytes have to make, so that they can deliver all essential hepatic functions while going through cell proliferation. The extensive "reprogramming" of hepatic gene expression requires activation of multiple signalling pathways involving matrix remodelling proteins, growth factors, cytokines, paracrine signals, and neuroendocrine factors.

Small RNAs, mainly microRNAs (miRNAs), provide an additional level of regulation in liver regeneration. Global loss of miRNAs leads to the impairment of

hepatocyte proliferation at the G1-S stage of cell cycle. In particular, miR-21, one of the upregulated miRNAs in HCCs, has been shown to increase the proliferation of hepatoma cells by targeting Pten and Btg2. As of now, data are limited and mainly restricted to the early phases of liver regeneration. Importantly, *in vivo* functions of individual miRNAs during liver regeneration have not yet been identified. Clearly, more work is required to further elucidate the functional role of known and novel miRNAs in all phases of liver regeneration including the termination process and to examine the effect of inhibition or over-expression of these miRNAs on liver regeneration.

The newborn liver contains mostly diploid hepatocytes, but polyploidization and binuclearity occur rapidly after birth. In perivenous areas hepatocytes are more often polyploid and serve different liver functions when compared to cells of the periportal region ("metabolic zonation") (Gorla et al. 2001; Jungermann and Kietzmann 2000). The gradient of less complex cells with higher proliferation potential (*in vitro*) in periportal areas and more mature hepatocytes in perivenous areas has been interpreted as evidence for the existence of a physiological niche for cell renewal (Sigal et al. 1995) in the periportal region. Recent experimental studies of hepatocytes with acquired mitochondrial mutations in the cytochrome c oxydase gene have also provided arguments for the periportal region as the "regenerative niche" in normal liver (Fellous et al. 2009). The "streaming liver hypothesis" postulating that the liver lobule is organized similar to the intestinal crypt and contains a stem/progenitor cell pool arising form the periportal area, however, has been disputed by gene marking studies (Bralet et al. 1994) and by the observation that nearly all hepatocytes proliferate as a response to injury regardless of location and ploidy.

Regenerative responses and cell types involved differ depending on the severity and chronicity of liver injury. Although mature hepatocytes and cholangiocytes represent the first and most important resource for tissue repair (Quante and Wang 2009; Duncan et al. 2009) and restore liver mass after acute toxic injury or surgical removal of liver mass, a liver stem/progenitor cell compartment is likely to be involved in the repair of chronically injured livers.

The first evidence for the existence and activation of a resident hepatic stem/progenitor cells compartment was provided by various murine animal models of "oval cell" proliferation (Alison et al. 1997; Fausto 2004; Thorgeirsson 1996). The general principle underlying "oval cell" activation is based on a combination of a liver injury and the inability of hepatocytes to proliferate in response to the damage. These "oval cells" most likely play a facultative role in liver regeneration, i.e., they contribute to tissue regeneration in cases, where adult hepatocyte proliferation is inhibited or exhausted (Fausto and Campbell 2003). Until now it is not known, whether "oval cells" pre-exist in the tissue or develop from mature adult cell types (i.e. bile duct cells) after an injury.

In parallel to what we know from rodent models, also in human liver diseases the inhibition of mature hepatocyte replication favors the proliferation of cell populations with stem/progenitor phenotypes. Activation of these cells has been associated with a variety of liver diseases, and, the numbers have been related to severity of the disease (Roskams et al. 2003; Roskams 2006). It has recently been shown

that hepatocytes become senescent, owing at least partially to telomere shortening, in the cirrhotic stage of a wide variety of chronic human liver diseases (Marshall et al. 2005; Wiemann et al. 2002). Replicative exhaustion and senescence of the mature hepatocytes as a result of ongoing proliferation during 20–30 years of chronic liver disease has been linked to the emergence of these stem/progenitor cells and finally with the evolution of hepatocarcinoma and cholangiocarcinoma (Alison and Lovell 2005; Mishra et al. 2009). However, it is unclear whether these cells are simply a marker of carcinogenic disease states or whether the stem/progenitor cells are at particular risk for transformation.

37.2 Regenerative Medicine in Hepatology

In this chapter we discuss three major areas in hepatology, for which regenerative therapies are being developed: "acute" and "acute on chronic" liver failure, chronic liver disease and hereditary (mostly monogenetic) liver diseases. The acute and self-limiting liver diseases (e.g. due to acute viral disease, toxins, transient ischemia) normally result in complete regeneration and "*restitutio ad integrum*". More massive injuries may temporarily exhaust the regenerative capacity of the liver and result in "acute liver failure", a clinical syndrome, which is characterized by progressive loss of hepatic function und multiorgan failure.

Persistent injuries to the liver also induce regenerative responses but eventually result in scarring and excess deposition of extracellular matrix components including collagen. Fibrosis and cirrhosis are the end result of chronic inflammatory reactions induced by a variety of stimuli including persistent infections, autoimmune reactions, allergic responses, chemical insults, radiation, and tissue injury. Although current treatments for fibrotic diseases such as idiopathic pulmonary fibrosis, systemic sclerosis and liver fibrosis/cirrhosis typically target the inflammatory response, there is accumulating evidence that the mechanisms driving liver fibrogenesis are distinct from those regulating inflammation. The key cellular mediator of fibrosis is the myofibroblast, which, once activated, serves as the primary collagen-producing cell. Myofibroblasts are generated from a variety of sources including resident mesenchymal cells, epithelial and endothelial cells in processes termed epithelial/endothelial-mesenchymal (EMT/EndMT) transition, as well as from circulating fibroblast-like cells called fibrocytes that are derived from bone-marrow stem cells. Myofibroblasts are activated by several mechanisms, including paracrine signals derived from lymphocytes and macrophages, autocrine factors secreted by myofibroblasts, and pathogen-associated molecular patterns (PAMPS) produced by pathogenic organisms that interact with pattern recognition receptors (i.e. TLRs) on fibroblasts.

The liver is central to many metabolic activities with hundreds of genes involved in their regulation. In recent years the genetic basis for more than 100 liver diseases involving malfunction of the organ has been clarified. Hereditary liver diseases usually result from point mutations, deletions or other genetic defects in single or multiple

genes, which are normally expressed in the liver and can cause acute and chronic liver diseases. The liver also secrets many proteins, which deliver functions for other organ systems, and a state of protein deficiency may not affect the liver function itself. For most of the hereditary liver diseases liver organ transplantation cures the disease or the state of protein deficiency and has become the most important therapeutic approach. Conceptually, many of these disorders, for which organ transplantation is effective, can be principally cured by cell- or gene therapies.

37.3 Cells for the Treatment of Liver Diseases

Many of the regenerative technologies generated or envisioned to treat liver diseases are based on cellular substrates, which are either transplanted/injected into recipients or utilized in extracorporeal devices. The primary hepatocyte, which can be isolated from adult liver organs, is still the most important cellular resource in clinical situations, in which specific liver functions need to be reconstituted. Hepatocytes from pig and human livers as well as immortalized human hepatocytes have been tested in extracorporeal liver devices (see Sect. 37.5.1.1). Transplanted human hepatocytes have been shown to engraft in the recipient liver and to respond to growth stimuli in vivo (Dandri et al. 2001; Bissig et al. 2007; Haridass et al. 2009). Despite a high proliferative capacity of hepatocytes, which can undergo more than 69 cell doublings or a 7.3×10^{20}-fold expansion (Overturf et al. 1997b) in vivo, the proliferation capacity in vitro is very restricted.

This lack of in vitro expansion protocols has stimulated the search for alternative cell sources, which can either expand in cell culture or can be easily harvested from the body in large quantities. Immortalized hepatocytes derived from adult and fetal tissue are restricted to ex vivo applications and have been applied in extracorporal liver devices (see Sect. 37.5.1.1). Human fetal liver derived hepatoblasts have been applied in a small number of patients with acute liver failure (Habibullah et al. 1994) and recently in one patient with hereditary bilirubinemia (Khan et al. 2008). These cells are also being tested as a cellular substrate for bioartificial liver devices (Poyck et al. 2008). Although the isolation of clinical grade stem/progenitor cells from human adult livers has been described, clinical applications have not yet been reported.

It has been proposed that (subpopulations of) adult hematopoietic stem cells (HSC), mesenchymal stromal cells (MSC) and cord blood stem cells (CBSC) can transdifferentiate into hepatocytes after transplantation, but the efficacy, by which these cells spontaneously form hepatocytes and liver tissue in animal experiments, still seems questionable (Petersen et al. 1999; Alison et al. 2000; Newsome et al. 2003; Aurich et al. 2007; Cantz et al. 2004) (Fig. 37.1). As an alternative concept, HSC, MSC and CBSC are being applied in patients with chronic liver disease with the therapeutic aim to induce liver regeneration and remodelling (see Sects. 37.5.3.2 and 37.5.2.1)

High expectations have been attributed to embryonic stem (ES) cells and more recently to induced pluripotent stem (iPS) cells. These cells can be maintained in a state of pluripotency for long periods of time, grown in large quantities (Evans and

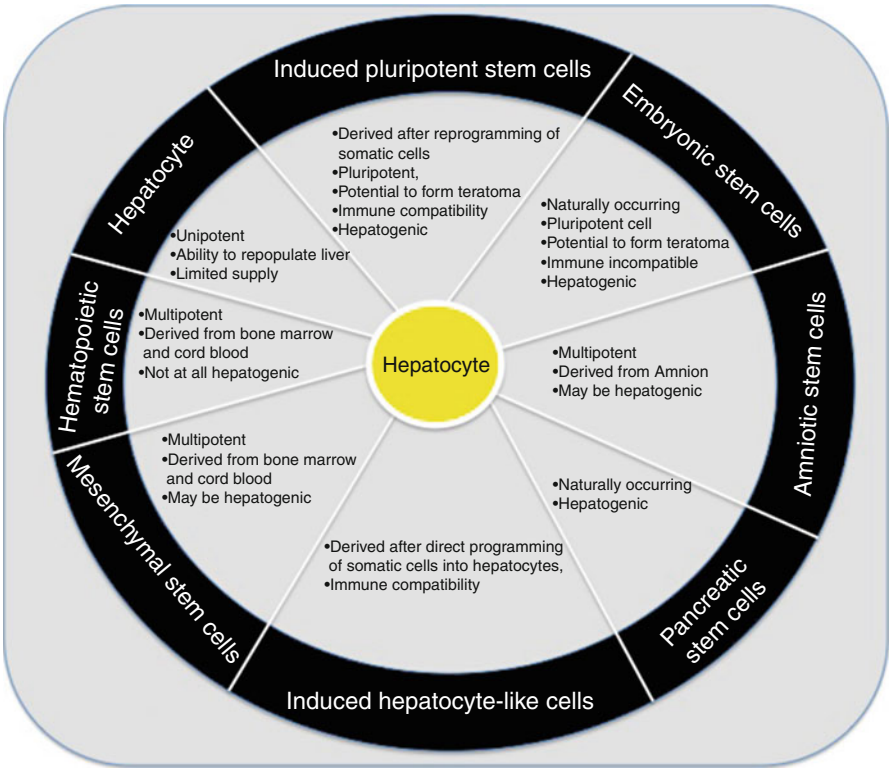


Fig. 37.1 Differentiation capacity and hepatogenic potential of cellular sources

Kaufman 1981; Rathjen and Rathjen 2001; Thomson et al. 1998; Boiani and Scholer 2005; Takahashi and Yamanaka 2006; Takahashi et al. 2007) and differentiated into virtually all cell types of the body. The direct transcription factor-mediated conversion of fibroblasts into hepatic cells, which could at least temporarily rescue a murine model of metabolic liver failure, was recently demonstrated by two independent groups (Huang et al. 2011; Sekiya and Suzuki 2011). To date, it remains speculative, whether direct "trans-programming" of adult stem cells or fibroblasts into the desired phenotype by forced expression of sets of transcription factors represents an alternative approach and may circumvent the state of pluripotency, which is associated with teratoma formation in transplanted recipients

37.3.1 Modes of Therapeutic Activity

Various modes of therapeutic activity have been proposed for transplanted cells. Transplanted primary hepatocytes, fetal hepatoblasts and adult liver progenitor cells engraft in the recipient liver and function as parenchymal liver cells. In vitro hepatic

differentiation protocols for stem cells, in particular embryonic and iPS cells, aim to generate cell phenotypes compatible with long term engraft in the liver and hepatocyte functionality.

Several ways to obtain therapeutic activity have been proposed for transplanted unmodified stem cells. For example, the injection of hematopoietic stem cells (HSC) isolated from adult bone marrow or cord blood was shown to generate hepatocytes at therapeutically significant levels in animal models (Petersen et al. 1999; Alison et al. 2000; Newsome et al. 2003). Initial studies suggested that those extrahepatic stem cells transdifferentiate from hematopoietic to hepatic lineage in the recipient organ, but more recent work has demonstrated fusion of stem cells with hepatocytes as the main mechanism (Wang et al. 2003). In an alternative concept HSC and mesenchymal stromal cells have been injected in animals and humans with chronic liver disease in order to induce regeneration and remodelling of the recipient liver without forming hepatocytes. Paracrine signalling of the transplanted cells and direct cell-cell contact have been proposed as main mechanisms in this setting.

37.3.2 Generation of Hepatocytes from ES/iPS Cells

Hepatocytes derived from ES cells may serve as an unlimited cell source unlike primary hepatocytes isolated from donor livers. In order to generate hepatocytes from pluripotent stem cells the various differentiation protocols usually mimic the events occurring during embryonic development of the liver. Accordingly, the pluripotent ES cells are differentiated into the hepatocyte state by the formation of embryoid bodies, followed by the induction of definitive endoderm using instructive cytokines such as Activin A. The endoderm cell population can be further induced towards the hepatocyte lineage by exposure to bone morphogenetic protein (BMP) 4 and fibroblast growth factor (FGF) 2, both important signals from the cardiac mesoderm in early liver embryogenesis (Gouon-Evans et al. 2006). Assessment of the hepatic phenotype is commonly based on hepatocyte specific gene expression profiles and metabolic activities such as cytochrome p450 activity, glycogen storage or urea synthesis which determine the efficacy of the differentiation protocol. At the end of a differentiation process it is important to remove contaminating undifferentiated ES cells from the heterogeneous cell culture to minimize the risk of teratoma formation. This can be achieved by various FACS/MACS sorting techniques or by the transfer of cell type specific expression of antibiotic resistance genes (Drobinskaya et al. 2008). Transplantation of ES derived hepatic cells to the liver results in engraftment as both, mature hepatocytes and bile duct epithelial cells (Gouon-Evans et al. 2006; Touboul et al. 2010). The level of liver repopulation obtained with hepatocyte-differentiated ESCs is very low, but can be increased somewhat when the cells are transplanted into MUP–urokinase plasminogen activator/severe combined immunodeficient (SCID) mice (Heo et al. 2006). To date, most published ESC differentiation protocols generate hepatocyte-like cells, but not the fully functional, mature, and transplantable equivalents of hepatocytes that are isolated from adult liver.

The pioneering work of Yamanaka and colleagues has paved the way for generation of embryonic stem cell like cells from almost any postnatal organ such as skin, liver and blood. These cells have been named induced pluripotent stem (iPS) cells. The iPS cells are generated from somatic cells by transduction with viral vectors expressing the stem cell genes *oct4*, *sox2*, *c-myc* and *klf4* (Takahashi and Yamanaka 2006; Takahashi et al. 2007). The combination of these four transcription factors was identified from initially 24 different transcription factors. Later, it was shown that these 4 factors were also sufficient for human somatic cell reprogramming. Subsequently, iPS cells were generated without viral integration (Stadtfeld et al. 2008). The precise mechanisms of reprogramming remain elusive. iPS cells resemble ES cells as they possess self-renewal capacity, the ability to differentiate into cells of ectoderm, mesoderm and endoderm and form teratomas after transplantation in mice. Similar to mouse ES cells, hepatocyte differentiation of mouse and human iPS cells has been documented recently. Notably the hepatocytes derived from mouse iPS cells had hepatocyte marker expression and metabolic activity similar to hepatocytes derived from mouse ES cells (Li et al. 2010; Si-Tayeb et al. 2010; Song et al. 2009; Sullivan et al. 2010)

37.4 Liver Tissue Engineering

Liver tissue engineering is a new and emerging field in which a functional liver system is created using isolated hepatocytes and/or other cells types to treat acute and chronic liver diseases. Under circumstances in which a small, but functional liver tissue system could be engineered to provide the equivalent biological function proportional to a few percent of a normal, well-functioning liver, it would be possible to correct many disease phenotypes that result from various forms of inherited metabolic deficiencies. It has been demonstrated in animal models, that sheets of liver tissue can be grown under the renal capsule or under the skin (Ohashi et al. 2007, 2010). Alternatively, hepatic tissues could be engineered ex vivo to produce therapeutic effects allowing this approach to become an effective modality for the treatment of acute liver failure. Three dimensional liver bioreactors, which are the main component of cell based liver support devices (see Sect. 37.5.1.1.2), may be considered as a "tissue engineering" approach. A much more complex and not yet achieved task will be the generation of transplantable liver tissue with a functional blood supply and biliary system.

37.5 Regenerative Therapies for Liver Disease

Regenerative therapies or treatments involving regenerative technologies are currently being developed for liver diseases of diverse etiologies. In acute liver failure syndromes, acute on chronic liver failure and non-function of transplanted livers

the therapeutic approaches aim to substitute liver function (synthesis of proteins, metabolism and detoxification) either by extracorporal support devices, transplantation of liver cells or by engineering and transplantation of functional liver tissue. In chronic liver diseases conventional drugs, cytokines, stem cell therapy and gene transfer techniques are being employed to specifically interfere with the inflammatory and profibrotic pathways. In hereditary metabolic liver disease the experimental and clinical approaches focus on substitution of defective genes and proteins by allogeneic transplantation of hepatocytes or by gene therapy. Therapies involving regenerative technologies such as (stem) cell therapies and gene transfer protocols, which emerge for liver cancer, viral infections and immune mediated liver diseases, are beyond the scope of this book chapter and have been reviewed elsewhere.

37.5.1 Acute Liver Failure

Acute liver failure (ALF) is a syndrome of diverse etiology, in which patients without previously recognized liver disease sustain a liver injury that results in rapid loss of hepatic function. Depending on the etiology and severity of the insult, some patients undergo rapid hepatic regeneration and spontaneously recover. However, nearly half of the patients with ALF require and undergo orthotopic liver transplantation or die. Even with optimal early management many patients with ALF develop a cascade of complications often presaged by the systemic inflammatory response syndrome, which involves failure of nearly every organ system. For those patients no satisfactory treatment exists other than liver transplantation. However, the number of donor livers available is limited and the outcome of liver transplantation for ALF is significantly lower than transplantation for chronic liver disease. Furthermore, many ALF patients are not placed on the transplant list due to exclusion criteria such as sepsis, psychiatric illness, and multi-system organ failure. Specialised treatment algorithms for the intensive care of patients with liver failure and the introduction of antioxidative drug treatments have already significantly improved the survival of affected patients in the past. Trials of plasmapheresis and hypothermia from European consortia are near completion and drugs that facilitate the excretion of ammonia, such as L-ornithine phenylacetate (Jalan et al. 2007), may provide a neuroprotective bridge to orthotopic liver transplantation.

37.5.1.1 Extracorporeal Liver Support in Acute and Acute-on-Chronic Liver Failure Patients

Future therapies for ALF would ideally maintain the patient's clinical stability long enough to allow liver regeneration to occur, which would obviate the need for orthotopic liver transplantation. Realistically, however, the goal of such therapies will be to serve as a bridge to orthotopic liver transplantation. Extracorporeal liver support

devices have been developed to achieve the goal of "bridging" by temporarily supporting liver detoxification function. Artificial liver support refers to purely mechanical devices including albumin dialysis, while bioartificial liver support refers to devices with a cellular component. Artificial systems remove toxins by filtration or adsorption while bioartificial liver systems perform these functions along with biotransformation and synthetic functions of biochemically active hepatocytes.

Non-cell Based Liver Support Devices

The molecular adsorbent recirculating system (MARS™; Gambro, Lund, Sweden) is the most frequently used type of albumin dialysis and most studied non-cell based liver-support technique (Mitzner et al. 2009). The key feature to the function of albumin dialysis is the concentration gradient of low-molecular-weight substrates between the patient's blood and the 20% albumin in the secondary circuit. This concentration gradient allows diffusible low-molecular-weight substrates to flow down their gradient over the membrane where they are transiently bound by albumin in the secondary circuit. (Steiner et al. 2004). The low-molecular-weight substrate is then removed from the system by conventional dialysis and hemodiafiltration within the secondary circuit.

The initial clinical study described a series of 13 patients who underwent treatment after failure of response to best medical therapy for acute-on-chronic hepatic failure. In this series, the overall survival was 69% and the authors cited that all patients showed a positive response to therapy (Stange et al. 1999). Other encouraging case reports and small studies eventually led to more widespread use of the system. To date, roughly 7,500 patients have been treated with MARS for various hepatic diseases, including acute liver failure patients. A meta-analysis assessing the use of MARS looked at four randomized controlled trials including a total of 67 patients and two selected nonrandomized trials including 61 patients (Khuroo et al. 2004). Patients had either acute or acute-on-chronic liver failure. Primary meta-analysis did not show a statistically significant survival benefit. Subgroup analysis for both acute and acute-on-chronic liver failure again failed to show a significant survival benefit. However, explorative analysis of the two nonrandomized trials did show that survival was significantly improved with MARS treatment. The authors concluded that this benefit was possibly related to bias in patient selection. Recently, the results of a large multicenter randomized trial of MARS in patients with ALF fulfilling high-urgency liver-transplant criteria in France were presented (Saliba et al. 2009). The data show a trend toward better surivival in the MARS treatment group, but the difference did not reach significance. The transplant-free 6 month survival, however, was significantly prolonged in those patients treated with at least three sessions of MARS. Although a relatively large number of patients have been treated with MARS and improvements in biochemical and physiologic parameters have been demonstrated, MARS must still be considered experimental, as survival benefit has not been reproducibly shown for the various indications.

"Prometheus"™, which employs fractionated plasma separation, is a close variant of MARS. While MARS is a two-circuit system separated by an albumin impermeable membrane, Prometheus utilizes a membrane with a 250 kDa cutoff between circuits, thereby making the membrane permeable to albumin and hence albumin-bound toxins. While a large portion of the toxins, which accumulate during liver failure are water soluble, many are still bound by albumin. Therefore fractionated plasma separation may be advantageous in regard to toxin removal. Other factors that distinguish Prometheus from MARS include the fact that while MARS is prefilled with 120 g of exogenous human albumin, the patient's endogenous albumin loads the secondary circuit in Prometheus. Because Prometheus is loaded with the patient's albumin, there may be a drop in the patient's albumin levels during treatment (Rifai et al. 2003; Santoro et al. 2006).

Most of the clinical data involving Prometheus are either uncontrolled or retrospective. A controlled trial, published as an abstract, looking at the effect of fractionated plasma separation on hepatic encephalopathy, demonstrated that a 6-h treatment course improved clinical grade and sensory-evoked potentials (Kramer et al. 2000). Multiple case series describe both acute and acute-on-chronic liver failure patients being treated with Prometheus. Only recently, the results of a controlled randomised multicenter clinical trial in 145 patients with acute on chronic liver failure were reported in abstract form (Rifai et al. 2010). Survival rates after 1 and 3 month were not significantly different in the treated versus the control group. However, patients with hepatorenal syndrome type I and MELD score of >30 showed a significant survival benefit. Currently available data thus illustrate a need for new prospective randomized controlled trials to clarify indications and clinical impact of extracorporeal artificial liver support devices.

Cell-Based Liver Support Devices

It is unlikely that the complex mechanism, by which the liver ensures homeostasis, can be replaced by means of nonbiologic detoxification alone. A bioartificial liver, which incorporates hepatocytes from various sources, has the theoretical advantage of not only providing blood purification through dialysis, but also providing the hepatocyte-specific functions which are lost with ALF. These include protein synthesis, ureagenesis, gluconeogenesis, and detoxification through P450 activity.

The first biologically based liver assist device to be tested in FDA-approved phase II/III trial was HepatAssist™ by Arbios (formerly Circe, Waltham, MA). The device employed a hollow fiber extracorporeal bioreactor loaded with cryopreserved primary porcine hepatocytes. A randomized, controlled, multicenter phase II/III clinical trial was conducted in patients with fulminant/subfulminant liver failure and primary graft nonfunction (Demetriou et al. 2004). The study demonstrated favorable safety, but failed to demonstrate improved 30-day survival in the overall study population. Although sub-groups of the study population showed significant survival benefits, HepatAssist is not yet approved by the FDA. The Extracorporeal Liver Assist Device (ELAD™) by Vital Therapies (San Diego, CA) utilizes hollow

fiber cartridges loaded with cells from the C3A human hepatoblastoma cell line. The most current model also contains a conventional hemodialysis unit. An early randomized controlled trial of 24 patients with acute alcoholic hepatitis demonstrated that therapy with ELAD produced reduced levels of ammonia and bilirubin along with improvement in hepatic encephalopathy when compared to controls (Ellis et al. 1999). However, a statistically significant survival advantage was not demonstrated. The Modular Extracorporeal Liver Support System (MELS™; Charité, Berlin, Germany) is a hepatocyte based liver support therapy composed of four independently functioning hollow fiber capillary cell compartments. A phase I study in 2003 including eight patients with ALF demonstrated safety, with all eight patients being successfully bridged to transplantation (Sauer et al. 2003). Clinical experience with MELS has been limited by the infrequent and unpredictable supply of human hepatocytes and concerns of xenozoonosis involving pig hepatocytes which are prevalent in Europe. The Bioartificial Liver Support System (BLSSTM) by Excorp Medical (Minneapolis, MN) is a system that utilizes ~100 g of primary porcine hepatocytes in a single hollow fiber cartridge. Venovenous bypass is used to circulate the patient's blood through the system. A phase I trial, in which four patients were treated with BLSS demonstrated safety (Mazariegos et al. 2001). Currently, a phase II/III study is underway, and results will further define the role of this device. The Amsterdam Medical Center bioartificial liver (AMC-BALTM; AMC, Amsterdam, The Netherlands) uses 100 g of primary porcine hepatocytes bound to a spiral-shaped polyester fabric with integrated hollow fibers. During treatment, the bioreactor is perfused with the patient's plasma. A phase I study of the system examined seven patients with ALF who underwent multiple treatments with AMC-BAL (Van De Kerkhove et al. 2002). Six were successfully bridged to transplantation, and one patient recovered liver function without transplantation. Improvements were observed in both clinical and biochemical parameters including a decrease in both bilirubin and ammonia. No adverse events were associated with treatment. While preliminary results were encouraging, larger randomized, controlled trials are needed to determine the role of AMC-BAL.

37.5.1.2 Hepatocyte and Stem Cell Transplantation

In acute liver failure hepatocyte transplantation may act as a bridge to recovery and regeneration of the injured native liver or alternatively to orthotopic liver transplantation once an organ becomes available. The procedure may also be used in patients who are not candidates for organ transplantation. A major advantage of hepatocyte transplantation is the immediate availability of cryopreserved cells. Sufficient cell mass (approximately 10–15% of liver cell mass) is needed to provide enough metabolic function (Asonuma et al. 1992). The mass of cells, which can be transplanted into the liver, is, however, limited by the effect on portal hypertension. Other options include intrasplenic or intraperitoneal transplantation, which allow a larger volume of cells. The spleen has been used successfully as injection site in animal (Kobayashi et al. 2000; Cai et al. 2002) and human transplantation (Bilir et al. 2000); however,

in view of the number of immunologically active cells located in the spleen, rejection or destruction of the non-native cells needs consideration. Hepatocyte transplantation in patients with ALF has resulted in a reduction in ammonia and bilirubin with improvements in hepatic encephalopathy and cardiovascular instability (Bilir et al. 2000; Fisher and Strom 2006). In the absence of any randomized controlled trials, it is difficult to comment on the true efficacy of the intervention.

There are a few studies on liver cell therapy for treatment of acute liver failure in humans with the intention to bridge the patients to orthotopic liver transplantation or recovery (Bilir et al. 2000; Schneider et al. 2006; Fisher and Strom 2006). Main challenges for future applications are the appropriate timing of cell transplantation, the restricted uptake capacity of the recipient liver, the availability of cells and the need for immunosuppression to prevent the rejection of the transplanted cells. The latter point may become more important than considered before, because the liver failure gives a high risk for septic complications itself, which will be aggravated by immunosuppressive drugs.

Extended liver resections have been associated with significant morbidity and mortality due to hepatic dysfunction or hepatic failure in the postoperative period. Autologous bone marrow stem cell therapies may offer the potential to enhance hepatic regeneration in this setting, perhaps increasing the safety of the procedure. Preclinical models and initial translational studies have suggested that autologous bone marrow stem cell administration can facilitate hepatic regeneration following both acute and chronic liver disease (Stutchfield et al. 2010). Infusion of HSC in three patients after extended liver resection demonstrated the therapeutic potential, however, more and controlled clinical trial data are needed (am Esch et al. 2005).

37.5.2 Chronic Liver Disease and Liver Fibrosis

Chronic injury and inflammation triggers a gradual loss of liver function and deposition of extracellular matrix components, which leads to fibrosis and finally to cirrhosis of the liver. Although acute injury does activate mechanisms of fibrogenesis, more sustained signals associated with chronic liver diseases lead to a fibrogenic response which engages several different cell types. Cirrhosis of the liver as a clinical endpoint of the fibrogenic process is probably an irreversible condition and the only long-term therapeutic solution for end-stage chronic liver disease today is liver organ transplantation. However, experimental and clinical data indicate that earlier events of the perpetuated fibrogenic process in the liver can be stopped or even reversed.

There is now experimental evidence that several endogenous factors/cytokines play important roles in regulation of liver fibrogenesis. The use of interferon alpha -2α and -2β is nowadays the main therapeutic strategy for the treatment of chronic viral hepatitis and compensated viral liver cirrhosis (Manns et al. 2001; Fried et al. 2002). In addition to decreasing viremia in HBV and HCV infections, it also leads to reduced liver fibrosis.

New therapeutic targets interfering with fibrogenesis are emerging from translational research and have been recently addressed in clinical trials. Interferon-gamma1β (IFN-γ1β) is a pleiotropic cytokine that displays antifibrotic, antiviral, and antiproliferative activity. Initial studies conducted in patients with HCV-related liver diseases have shown a fibrosis reduction in some of the patients (Muir et al. 2006). In particular, patients with elevated interferon-inducible T cell-alpha chemoattractant (ITAC) levels in their blood and perhaps less advanced disease stage, may best be suited for IFN-gamma1β based therapy (Pockros et al. 2007).

Interleukin-10 (IL-10) was first described as a cytokine synthesis inhibitory factor for T lymphocytes produced from T helper 2 cell clones. In fact various cell populations produce IL-10 in the body, including T cell subsets, monocytes, macrophages and also various other cell types present in organs such as the liver. IL-10 gene polymorphisms are possibly associated with liver disease susceptibility or severity. Recombinant human IL-10 is currently tested in clinical trials in patients not responding to standard Peg-IFN α therapy.

PDGF is the most potent mitogen for hepatic stellate cell-derived myofibroblasts and levels of the growth factor have been shown to increase in liver diseases. Autocrine signalling by PDGF was the first cytokine loop discovered in hepatic stellate cell activation and is amongst the most potent ones (Borkham-Kamphorst et al. 2008). Hepatic PDGF-α overexpression using the CRP-gene promoter was accompanied by a significant increase in hepatic procollagen III mRNA expression as well as TGF-β1 expression. Liver histology showed increased deposition of extracellular matrix in transgenic but not in wildtype mice. These results point to a mechanism of fibrosis induction by PDGF-α via the TGF-β1 signalling pathway (Thieringer et al. 2008). On the other hand, Dominant-negative soluble PDGF receptor beta is currently investigated as a possible new antifibrogenic target.

TGFβ1 remains, however, the classic fibrogenic cytokine. TGF β1 activates stellate cells via the SMAD proteins pathway and also stimulates collagen expression in stellate cells through a hydrogen peroxide and C/EBPβ -dependent mechanism. There is experimental evidence that hepatocyte-specific overexpression of TGFβ1 in transgenic mice increases fibrosis *in vivo*, and that soluble TGFβ receptor type II treatment inhibits fibrosis *in vivo*. Also, it has been shown that adenovirus encoding antisense TGFβ mRNA inhibits fibrogenesis *in vivo*.

More experimental strategies aim to reduce extracellular matrix deposition by over-expression of MMP's. Siller-Lopez et al. have used an extrahepatic human neutrophil collagenase complementary MMP-8 DNA cloned in an adenovirus vector (AdMMP8) as a therapeutic agent in cirrhosis using CCl_4 and bile duct–ligated cirrhotic rats models. Liver fibrosis in bile duct–ligated cirrhotic animals was decreased by 45% along with reduced hydroxyproline levels in AdMMP8 treated animals. Treatment in both models correlated with improvements in ascites, functional hepatic tests and gastric varices indicating diminished portal hypertension in animals injected with AdMMP8 (Siller-Lopez et al. 2004).

Alternative treatment concepts aim to protect existing hepatocytes and/or to increase the hepatocyte mass. Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes (Nakamura et al. 1984, 1989: Russell

et al. 1984; Miyazawa et al. 1989) has mitogenic and morphogenic activities for a wide variety of cells (Boros and Miller 1995; Michalopoulos and DeFrances 1997) and also plays an essential role in the development and regeneration of the liver (Schmidt et al. 1995). It has also been shown to have antiapoptotic activity in hepatocytes (Bardelli et al. 1996). Transduction of the HGF gene has suppressed the increase of transforming growth factor-β1 (TGF-β1), which plays an essential part in the progression of liver cirrhosis and inhibited fibrogenesis and hepatocyte apoptosis leading to complete resolution of fibrosis in the cirrhotic liver in a rat model (Ueki et al. 1999).

37.5.2.1 Stem Cell Therapy of Chronic and Acute of Chronic Liver Disease

Although the concept of cell therapy for various diseases is principally accepted, the practical approach in humans remains difficult. Bone marrow derived mononucleated cells, hematopoietic stem and progenitor cells, mesenchymal stem (stromal) cells and sinusoidal endothelial cells are currently being investigated. There are several proposed mechanisms by which stem and progenitor cells might support regeneration in targeted organs including the liver: intercellular signalling through cell-cell contacts, paracrine signalling (growth factors, cytokines, hormones) or cell fate change in the target organ.

The concept of stem/progenitor cell infusions exerting a paracrine regenerative effect on the liver is gaining support and is backed up by both rodent and human studies, although the latter are small and uncontrolled. Endothelial precursor cells (EPC) have been shown in rodent models to promote angiogenesis and the degradation of liver scar tissue thereby contributing to liver regeneration (Taniguchi et al. 2006; Nakamura et al. 2007; Ueno et al. 2006). By participation in neovascularisation and by the expression of multiple growth factors, transplanted EPCs significantly accelerate liver regeneration. This is achieved by enhancing proliferative activity of hepatocytes leading to improved survival after chemically induced liver injury (Taniguchi et al. 2006).

Sakaida et al. have demonstrated that transplanted bone marrow cells degrade extracellular matrix in carbon tetrachloride (CCl_4)-induced liver fibrosis, with a significantly improved survival rate in this animal model. Their findings suggest that transplanted bone marrow cells can degrade collagen fibers and reduce liver fibrosis by strong expression of MMPs, especially MMP-9 (Sakaida et al. 2004).

Other groups have raised concerns about the role of certain subtypes of bone marrow stem cells in liver fibrogenesis (Russo et al. 2006). It has been shown that bone marrow derived myofibroblasts significantly contributed to fibrogenesis in a chronic liver injury model in mice. They originated predominantly from bone marrow cells enriched for mesenchymal progenitor cells. These cells were located in the region of hepatic scarring and actively expressed collagen. The data suggest that an axis of recruitment from the bone marrow to the liver does exist in chronic injury and that the therapeutic application of certain subsets of bone marrow derived cells may contribute to, rather than resolve scarring of the liver tissue. The choice of

the transplanted bone marrow cell type might thus be important with regard to supporting liver regeneration or fibrogenesis.

Taken together the infusion of stem cells might provide an array of factors supporting not only liver regeneration but also the remodelling of impaired liver architecture by interfering with fibrogenesis. Important experimental findings, however, suggest that infused bone marrow cells may also contribute to fibrogenesis (Takezawa et al. 1995: Kisseleva et al. 2006) giving some cautious notes for the uncritical use of stem cells for chronic liver disease outside of controlled clinical trials (Sakaida et al. 2004; Fang et al. 2004; Zhao et al. 2005; Oyagi et al. 2006).

Several clinical trials already investigated the effect of bone marrow (stem) cells in patients with liver disease. They were mainly uncontrolled, with only small numbers of patients enrolled and have provided heterogeneous results. The trials can be categorized in 4 groups according to the main endpoint and source of cells: (1) effects of granulocyte colony–stimulating factor (G-CSF) mobilized bone marrow cells in advanced chronic liver disease, (2) effects of infusion of autologous mononuclear cells collected from bone marrow in advanced chronic liver disease, (3) effects of collection (with or without ex vivo manipulation) and infusion of mobilized bone marrow cells in advanced chronic liver disease and (4) effects of bone marrow infusions on liver regeneration (after selective portal venous embolization) prior to extended hepatectomy for liver tumors (Houlihan and Newsome 2008; Gaia et al. 2006; Terai et al. 2006; Mohamadnejad et al. 2007a, b; Lyra et al. 2007a, b; Gordon et al. 2006; Levicar et al. 2008; Yannaki et al. 2006; am Esch et al. 2005; Pai et al. 2008)

The trials are quite heterogeneous with regard to the source of stem cells used and the number of patients included. The following stem cells sources have been used: bone marrow from iliac crest (50–400 ml), G-CSF mobilization only, G-CSF mobilization followed by leukapheresis and CD-34+ selection and reinfusion. All but one trial were non-randomized. The stem cells were administered by peripheral vein infusions (three studies), by hepatic artery infusions (five studies) or portal vein infusions (two studies). The largest study conducted so far by Lyra et al. was also the only randomised one and included 30 patients.

Eight out of 11 trials have shown a moderate improvement in liver function (albumin, INR, bilirubin, Child-Pugh score, MELD score) and the follow-up period has ranged from 2 to 12 months.

In one recent study safety and efficacy of hepatic artery administration of mobilized autologous and *ex vivo* expanded adult $CD34^+$ hematopoietic stem cells in patients with alcoholic cirrhosis (ALC) was assessed (Pai et al. 2008). This study reported one of the largest numbers of CD34 positive stem cells infused in cirrhotic patients so far. Nine patients with biopsy-proven ALC and abstinence from alcohol for at least 6 months were included in the study and all patients tolerated the procedure well, with no treatment-related side effects or toxicities observed. Significant improvement in liver function was shown by decrease in serum bilirubin levels, serum alanine transaminase and aspartate transaminase. The Child-Pugh score improved in seven out of nine patients and in five patients ascites production had declined.

Two studies so far aimed to ameliorate acute on chronic liver disease by administration of granylocyte – colony stimulating factor (G-CSF) treatment. In contrast to an earlier study by Di Campli et al. (2007) a more recent study from India showed profound effects on short term survival, which was associated with a marked increase of CD34 stem cells in the liver of recipients (Garg et al. 2012).

37.5.3 Hereditary Liver Disease

Liver organ transplantation can be viewed as a form of gene therapy for inherited liver diseases since the procedure substitutes a defective gene with a normal copy from a healthy donor. Animal studies have shown that for most monogenetic liver diseases partial substitution of a missing or defective protein is able to reverse the clinical phenotype and can result in complete remission of the disease. This redundancy opens the possibility to apply minimally invasive therapies such as cell and gene therapies to correct an existing gene defect. Although many hurdles still exist, feasibility has been proven unequivocally in animal models and therapeutic protocols are now emerging in the clinical arena.

37.5.3.1 Transplantation of Mature (Adult) Hepatocytes

In recent years the interest in liver cell therapy has been increasing continuously, since the demand for whole liver transplantations in human beings far outweighs the supply (Nussler et al. 2006). From the clinical point of view, transplantation of hepatocytes or hepatocyte-like cells may represent an alternative to orthotopic liver transplants for the correction of genetic disorders resulting in metabolically deficient states. The aim of hepatocyte transplantation in metabolic disease is to partially replace the missing function without the need to replace the whole organ. Almost 30 children and adults who received liver cell therapy for metabolic liver disease are reported in literature (Fisher and Strom 2006; Fitzpatrick et al. 2009). Clinical therapies up to now have been performed by infusing fresh or cryopreserved primary hepatocyte suspensions isolated from donated organs. The availability of high quality liver tissue for cell isolation, however, has slowed the widespread application of this therapy. Furthermore, the clinical situation of target patients is rarely immediately life threatening and often acceptable conventional therapies are available. Therefore, the potential benefit must be carefully weighed against any possible complications, such as side effect from immunosuppression, hepatocyte embolisation of the pulmonary vascular system, sepsis or hemodynamic instability.

Objective parameters such as laboratory data (i.e. bile acid, clotting factors, etc.) can be determined to unequivocally assess the efficacy of the treatment. The results of hepatocyte transplantation for many metabolic liver diseases have been encouraging with demonstrable, although short-term correction of metabolic deficiency in

the majority of cases. Therapeutic benefit has been reported in a girl with Crigler–Najjar Syndrome Type I, which is a recessively inherited metabolic disorder characterized by severe unconjugated hyperbilirubinaemia (Fox et al. 1998). Isolated hepatocytes were infused through the portal vein and partially corrected plasma bilirubin levels for more than 11 months. Similarly, a 9-year-old boy received 7.5×10^9 hepatocytes, infused via the portal vein, which resulted in a decrease in bilirubin level from 530 ± 38 μmol/L (mean ± SD) before to 359 ± 46 μmol/L (Ambrosino et al. 2005). Hughes et al. also report a 40% reduction in bilirubin levels in a Crigler–Najjar Syndrome Type I patient following transplantation of hepatocytes (Hughes et al. 2005). Although these data demonstrate efficacy and safety, a single course of cell application seems not sufficient to correct Crigler–Najjar Syndrome Type I completely.

Sustained response was reported in a patient with argininosuccinate lyase deficiency after repeated hepatocyte transplantation. Engraftment of the transplanted cells was analyzed in repeated liver biopsies for more than 12 month by fluorescence in situ hybridization for the Y-chromosome and by measurement of tissue enzyme activity (Stephenne et al. 2006). Promising results have also been obtained in a 47-year-old woman suffering from glycogen storage disease type 1a, an inherited disorder of glucose metabolism resulting from mutations in the gene encoding the hepatic enzyme glucose-6-phosphatase (Muraca et al. 2002). 2×10^9 ABO-compatible hepatocytes were infused into the portal vein. Nine months after cell transplantation, her metabolic situation had clearly improved. Successful hepatocyte transplantation has also been achieved in a 4-year-old girl with infantile Refsum disease, an inborn error of peroxysome metabolism, leading to increased levels of serum bile acids and the formation of abnormal bile acids (Sokal et al. 2003). A total of 2×10^9 hepatocytes from a male donor were given during eight separate intraportal infusions. Abnormal bile acid production (for instance pipecholic acid) had decreased by 40% after 18 months. Recently, hepatocyte transplantation has been used successfully to treat inherited factor VII deficiency (Dhawan et al. 2004). Two brothers (aged 3 months and 3 years) received infusions of 1.1 and 2.2×10^9 ABO-matched hepatocytes into the inferior mesenteric vein. Transplantation clearly improved the coagulation defect and decreased the necessity for exogenous factor VII to approximately 20% of that prior to cell therapy. As with the other metabolic liver diseases, hepatocyte transplantation has been shown to provide a partial correction of urea cycle defects. Patients showed clinical improvement, reduced ammonia levels and increased production of urea (Horslen et al. 2003: Mitry et al. 2004; Stephenne et al. 2005; Meyburg et al. 2009)

37.5.3.2 Transplantation of Stem Cells

In the last few years, many reports have suggested that extrahepatic stem cells participate in liver regeneration and may be useful for treating many diseases (Alison et al. 2000; Herzog et al. 2003; Lagasse et al. 2000; Petersen et al. 1999; Theise et al. 2000). However, subsequent work by several independent groups has

clearly shown that hepatocyte replacement levels after injection of extrahepatic stem cells or by bone marrow transplantation are low (<0.01%), unless those bone-marrow-derived hepatocytes have a selective growth advantage (Cantz et al. 2004; Kanazawa and Verma 2003; Wagers et al. 2002). Furthermore, in most of the cases, fusion with host hepatocytes rather than transdifferentiation of extrahepatic cells, has been described as the underlying mechanism (Alvarez-Dolado et al. 2003; Quintana-Bustamante et al. 2006; Vassilopoulos et al. 2003; Willenbring et al. 2004).

So far no convincing evidence has yet been provided in animal models that stem cells including HSC, MSC, iPS or cells derived from cord blood or the amnion can generate therapeutically significant numbers of hepatocytes for the correction of hereditary metabolic liver diseases. Consequently, no credible data on the use of stem cells in patients with hereditary liver disease have been published.

37.5.3.3 Gene Therapy

The liver is involved in the synthesis of serum proteins, regulation of metabolism and maintenance of homeostasis and thus provides a variety of opportunities for gene therapeutic corrections. Gene therapy is the treatment of an inherited or acquired disease through the manipulation of a patients' genetic status or sequence in selected cells by introducing various types of genetic materials such as virally bound nucleic acids, plasmid DNAs, antisense oligonucleotides and short interference RNAs. Both viral and non-viral methods have been developed for effective gene delivery. Currently, only viral vectors have transduction efficacies needed for liver-based gene therapy of inherited metabolic diseases in humans.

Viral Vectors

Viral gene delivery employs replication deficient viruses as a carrier to bring genetic materials into cells through their natural infection mechanism. Viral vectors are created using molecular biological techniques by which portion of the viral genome is replaced with a gene of interest. Major drawbacks of viral vectors are their genetic and immunologic toxicities, which are mainly associated with an arbitrary recombination with genomic DNA of the target cells and acute immune stimulation, respectively. Because adult humans have already developed immunity against several types of viruses from which viral vectors are developed, an exposure of the viral vectors to patients often results in strong immunological reaction, and consequently disables efficient gene delivery and long-term gene expression.

Viral vectors frequently used in gene therapy studies are derived from retrovirus, adenovirus, and adeno-associated virus. Retroviruses, enveloped RNA viruses with a particle size of approximately 100 nm, only infect dividing cells and are capable of integrating reverse transcribed DNA into the host genome at an unpredictable location (Sinn et al. 2005). Viral integration has led to leukemia development as

revealed by recent gene therapy trial on X-linked SCID (Bey et al. 2003). The requirement of hepatectomy (~70%) to stimulate hepatic proliferation is generally considered as a drawback for retrovirus mediated gene delivery to the liver (Rettinger et al. 1993; Branchereau et al. 1994). Lentiviruses, a subclass of retroviruses including human immunodeficiency virus, can transduce non-dividing as well as dividing cells. The lentivirus preintegration complex is able to pass the intact nuclear membrane, which allows it to integrate into the host genome without cell division (Amado and Chen 1999). It was reported that animals can be repeatedly infected with lentiviral vectors (Kafri et al. 1997).

Adenoviruses are double-stranded DNA viruses with a diameter of approximately 110 nm. Adenoviruses infect both replicating and non-replicating cells, have a relatively large genome, and are unable to integrate into the host genome (Ghosh et al. 2006b). A number of serotypes has been used to create adenoviral vectors and employed in 24.8% of clinical trials till the end of March, 2008 (*The Journal of Gene Medicine* 2008). These vectors exhibit a broad range of liver tropism with serotype 5 as the most commonly used to date (Jager and Ehrhardt 2007). Adenoviral vector is the first proven gene carrier for the treatment of cancer (Peng 2005). Because this virus is a natural human pathogen, preexisting immunity against adenovirus can cause severe allergic reaction and inactivation of viral vectors (Marshall 1999). The current strategy in avoiding these problems is to use a serotype which the patients have no immunity against (Jager and Ehrhardt 2007). If the immunogenic drawbacks can be overcome in the future, adenoviral vectors will probably find a great diversity of clinical applications.

Adeno-associated virus (AAV) belongs to the Parvoviridae virus family and is approximately 26 nm in diameter without envelope (Grieger and Samulski 2005). It requires a helper virus for replication such as adenovirus. It is non-pathogenic and can infect quiescent cells. AAV is currently classified into 12 serotypes, and the liver is known to be a preferential target especially for AAV-8 (Wu et al. 2006). It was reported that this virus can insert its genome at a defined site on chromosome 19 termed AAVS1 with nearly 100% certainty (Samulski et al. 1991) The site-directed integration is controlled by viral Rep proteins (Young et al. 2000), which are often deleted in recombinant AAV vectors in favour of more space for the exogenous gene to be packaged into the tiny viral particle. Cotransfection of plasmids coding for Rep protein was reported to restore capability of the site-directed integration and enable a long-term expression of the transgene without inducing insertional mutagenesis (Howden et al. 2007). Results from a number of animal studies also indicate that AAV is less immunogenic when compared to adenoviruses (Coura Rdos and Nardi 2007).

Preclinical Evaluation

Feasability of gene therapies has been demonstrated in a wide variety of animal models. Long-Evans cinnamon rats are a model of Wilson disease and transfer of the *ATP7B* gene to hepatocytes ameliorates both biochemical and histological

pathologies (Merle et al. 2006). Transgene products released into blood circulation after successful gene transfer into the hepatocytes corrected pathological manifestation both inside and/or outside of the liver in glycogen storage diseases (type Ia, (Ghosh et al. 2006a, b) Ib (Yiu et al. 2007) and II (Yiu et al. 2007)), mucopolysaccharidosis type I (Kobayashi et al. 2005), IIIB (Di Natale et al. 2005) and VII (Ponder et al. 2002), hereditary tyrosinemia type I (Overturf et al. 1996), UDP glucuronyltransferase deficiency (Crigler-Najjar type I) (Seppen et al. 2003), and hemophilia (Miao et al. 2001; Herzog et al. 1999; Waddington et al. 2004).

A complete and persistent phenotypic correction of phenylketonuria in mice was reported after hydrodynamic gene delivery of murine phenylalanine hydroxylase cDNA with the help of phiBT1 phage integrase for long-term gene expression (Chen and Woo 2005). Further, the efficacy of adenovirus-mediated *in vivo* gene therapy for ornithine transcarbamylase deficiency was reported in mice and non-human primates (Raper et al. 1998). Hyperlipidemia was not only effectively treated in the respective genetic mouse models through delivery of apolipoprotein B (Crooke et al. 2005) or E (Kim et al. 2001) genes but also in wild type mice treated with a high-fat diet. A reciprocal pathophysiological condition of hypoalphalipoproteinemia was effectively reversed by adenoviral transduction of human apolipoprotein A-I gene in model mice as well (Oka et al. 2007).

Liver-Directed Gene Therapy in Humans

Gene therapy has the potential to offer a definitive cure for monogenic diseases by achieving a long-term correction of pathology. Monogenic diseases in the liver are divided into two groups depending on whether cell damage in the liver is involved or not. For example, hemophilia, familiar hypercholesterolemia, and phenylketonuria show systemic manifestations without significant liver cell damage, and have the least risk for hepatotoxicity in orthotopic gene delivery. In fact phase I/II clinical trials for hemophilia B were completed with promising results (Manno et al. 2006). Unfortunately, however, the development of inhibitory antibodies against the exogenous factor IX and/or components of viral vectors diminished a persistent phenotypic correction (Manno et al. 2006; Mingozzi and High 2007). One possible solution to avoid antibody development against exogenous gene products is gene delivery into the fetal liver to induce tolerance to the exogenous gene products (Seppen et al. 2003; Waddington et al. 2004; Sabatino et al. 2007) or alternative injection routes (Tominaga et al. 2004). It is important to point out that significant difference exists between animal studies and human clinical trials with respect to immunological reactions (Ye et al. 2004; Gao et al. 2006).

In case of the monogenic liver diseases with substantial hepatocellular damage, gene therapy should not be a primary indication unless gene delivery can be completed in all hepatocytes in the liver. Successful delivery of human fumarylacetoacetate hydrolase gene into hepatocytes protected $FAH^{(-/-)}$ – mice mimicking hereditary tyrosinemia type 1 disease from fulminant liver failure by restoring the enzyme activity (Overturf et al. 1997a; Grompe et al. 1998). However, hepatocellular

damage continued in the rest of hepatocytes that had not received the transgene and resulted in the frequent development of hepatocellular carcinoma. Liver transplantation should be a primary option for the diseases in this category at this moment.

37.6 Advanced Therapy Medicinal Products Regulation

For many of the regenerative therapies discussed in this chapter a new legal framework on advanced therapy medicinal products (ATMPs), which was implemented by the European Medicine Agency (EMEA) in December, 2008, is now applicable (Regulation (EC) No 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending directive 2001/83/EC and regulation (EC)No 726/2004. Official Journal of the European Union (10.12. 2007) L324/121). ATMPs are defined as "innovative, regenerative therapies which combine aspects of medicine, cell biology, science and engineering for the purpose of regenerating, repairing or replacing damaged tissues or cells" and fall into three categories: (a) gene therapy 'medicinal products' (b) somatic cell therapy 'medicinal products' or (c) tissue engineered products. As a result of this European Union legislation, the EMEA has introduced the Committee for Advancced Therapies (CAT). The CAT will play a central role in safety and efficacy assessment of new ATMP's prior to formal marketing approval. This central European legislation will surely facilitate the development and wide spread application of regenerative therapies in hepatology and other medical disciplines.

37.7 Future Directions

Regenerative therapies involving various types of cells as well as gene therapies are currently being investigated in research laboratories around the world and more and more find the way into therapeutic algorithms in the clinic. Bioartificial liver support systems and cell therapies are currently limited by the availability of good quality hepatocytes. A renewable source of highly metabolically competent hepatocytes will be essential for any successful bioartificial liver system. To date porcine hepatocytes are most commonly being used with limited acceptance due to ongoing concerns of xenozoonosis. Immortalized human hepatocytes have not shown expression of prerequisite hepatocyte function including ammonia detoxification. Other limitations of first-generation bioartificial liver systems, which need to be solved, include excess device complexity, insufficient number of hepatocytes to support a failing liver, early hepatocyte death, and absence or loss of differentiated function.

The application of stem cells in liver cell therapies seems to be a promising approach for the treatment of liver diseases. However, several issues still have to be addressed to fulfil this promise. We need to identify, both inside and outside of the liver, the stem cell candidates that are able to form mature hepatocytes in vitro and

functional liver tissue after transplantation in vivo. The fundamental molecular pathways involved in the differentiation of hepatocytes and cholangiocytes from stem/progenitor cells, the factors that are responsible for in vitro differentiation of various stem cells into hepatocytes, the mechanisms involved in the fusion of stem cells and hepatocytes and the aspects that can potentially enhance these mechanisms need to be studied in more detail. With future progress in stem cell research, the various stem cell sources including hepatic stem/progenitor cells, embryonic and adult extrahepatic stem cells should provide great opportunities for the treatment of liver disorders.

Additional work is also needed in the development of an ideal gene delivery system. The efficacy of delivery and the level of transgene expression achieved by the current methods have resulted in phenotypic correction of various hereditary liver diseases in animal models. The most efficient vehicles for gene delivery to the liver developed so far are viral vectors. Among the viral vectors applicable to liver gene delivery, lentiviral vectors appear to have great advantage because of their ability to transduce the liver cells at resting state and generate persistent gene expression. Gene toxicity by insertional mutagenesis with the transactivation of potentially harmful genes and interactions of the host immune system with the viral proteins and the therapeutic product need to be studied in more detail. Active participation of hepatologists in gene therapy research will accelerate the process in turning gene therapy into a common practice for the treatment of various diseases through the liver.

In summary, advanced approaches in regenerative hepatology will cover strategies to improve endogenous liver regeneration, to correct monogenetic liver diseases by gene therapy, and to support organ function with additional hepatic cells, either in extracorporal devices or as cell transplants. For the latter aspect, improved cell isolation and propagation techniques to utilize cells from donor organs or advanced stem cell-differentiation protocols become of utmost importance to ensure the supply of functional hepatic cells.

References

Alison MR, Lovell MJ (2005) Liver cancer: the role of stem cells. Cell Prolif 38:407–421

Alison M, Golding M, Lalani EN, Nagy P, Thorgeirsson S, Sarraf C (1997) Wholesale hepatocytic differentiation in the rat from ductular oval cells, the progeny of biliary stem cells. J Hepatol 26:343–352

Alison MR, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M, Prentice G, Williamson J, Wright NA (2000) Hepatocytes from non-hepatic adult stem cells. Nature 406:257

Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425:968–973

am Esch JS 2nd, Knoefel WT, Klein M et al (2005) Portal application of autologous CD133+ bone marrow cells to the liver: a novel concept to support hepatic regeneration. Stem Cells 23:463–470

Amado RG, Chen IS (1999) Lentiviral vectors – the promise of gene therapy within reach. Science 285:674–676
Ambrosino G, Varotto S, Strom SC, Guariso G, Franchin E, Miotto D et al (2005) Isolated hepatocyte transplantation for Crigler–Najjar syndrome type 1. Cell Transplant 14:151–157
Asonuma K, Gilbert JC, Stein JE, Takeda T, Vacanti JP (1992) Quantitation of transplanted hepatic mass necessary to cure the Gunn rat model of hyperbilirubinemia. J Pediatr Surg 27:298–301
Aurich I, Mueller LP, Aurich H, Luetzkendorf J, Tisljar K, Dollinger MM, Schormann W, Walldorf J, Hengstler JG, Fleig WE, Christ B (2007) Functional integration of hepatocytes derived from human mesenchymal stem cells into mouse livers. Gut 56(3):405–415
Bardelli A et al (1996) HGF receptor associates with anti-apoptotic protein BAG-1 and prevents cell death. EMBO J 15:6205–6212
Bey H, Abina S, von Kalle C et al (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 348:255–256
Bilir BM, Guinette D, Karrer F et al (2000) Hepatocyte transplantation in acute liver failure. Liver Transpl 6:32–40
Bissig KD, Le TT, Woods NB, Verma IM (2007) Repopulation of adult and neonatal mice with human hepatocytes: a chimeric animal model. Proc Natl Acad Sci USA 104(51):20507–20511
Boiani M, Scholer HR (2005) Regulatory networks in embryo-derived pluripotent stem cells. Nat Rev Mol Cell Biol 6:872–884
Borkham-Kamphorst E, Kovalenko E, van Roeyen CR, Gassler N, Bomble M et al (2008) Platelet-derived growth factor isoform expression in carbon tetrachloride-induced chronic liver injury. Lab Invest 88(10):1090–1100
Boros P, Miller CM (1995) Hepatocyte growth factor: a multifunctional cytokine. Lancet 345:293–295
Bralet MP, Branchereau S, Brechot C, Ferry N (1994) Cell lineage study in the liver using retroviral mediated gene transfer: evidence against the streaming of hepatocytes in normal liver. Am J Pathol 144:896–905
Branchereau S, Calise D, Ferry N (1994) Factors influencing retroviral-mediated gene transfer into hepatocytes in vivo. Hum Gene Ther 5:803–808
Burt AD, Portmann BC, Ferrell LD (2007) MacSween's pathology of the liver. Elsevier, Philadelphia
Cai J, Ito M, Nagata H et al (2002) Treatment of liver failure in rats with end-stage cirrhosis by transplantation of immortalized hepatocytes. Hepatology 36:386–394
Cantz T, Sharma AD, Jochheim-Richter A, Arseniev L, Klein C, Manns MP, Ott M (2004) Reevaluation of bone marrow-derived cells as a source for hepatocyte regeneration. Cell Transplant 13:659–666
Chen L, Woo SL (2005) Complete and persistent phenotypic correction of phenylketonuria in mice by site-specific genome integration of murine phenylalanine hydroxylase cDNA. Proc Natl Acad Sci USA 102:15581–15586
Coura Rdos S, Nardi NB (2007) The state of the art of adeno-associated virus-based vectors in gene therapy. Virol J 4:99
Crooke RM, Graham MJ, Lemonidis KM, Whipple CP, Koo S, Perera RJ (2005) An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis. J Lipid Res 46:872–884
Dandri M, Burda MR, Török E, Pollok JM, Iwanska A, Sommer G, Rogiers X, Rogler CE, Gupta S, Will H, Greten H, Petersen J (2001) Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. Hepatology 33(4):981–988
Demetriou AA, Brown RS Jr, Busuttil RW (2004) Prospective, randomized, multicenter, controlled trial of a bioartificial liver in treating acute liver failure. Ann Surg 239:660–667
Dhawan A, Mitry RR, Hughes RD, Lehec S, Terry C, Bansal S et al (2004) Hepatocyte transplantation for inherited factor VII deficiency. Transplantation 78:1812–1814
Di Campli C, Zocco MA, Saulnier N, Grieco A, Rapaccini G, Addolorato G, Rumi C, Santoliquido A, Leone G, Gasbarrini G, Gasbarrini A (2007) Safety and efficacy profile of G-CSF therapy in patients with acute on chronic liver failure. Dig Liver Dis 39:1071–1076

Di Natale P, Di Domenico C, Gargiulo N et al (2005) Treatment of the mouse model of mucopolysaccharidosis type IIIB with lentiviral-NAGLU vector. Biochem J 388:639–646
Drobinskaya I, Linn T, Saric T, Bretzel RG, Bohlen H, Hescheler J, Kolossov E (2008) Scalable selection of hepatocyte- and hepatocyte precursor-like cells from culture of differentiating transgenically modified murine embryonic stem cells. Stem Cells 26(9):2245–2256
Duncan AW, Dorrell C, Grompe M (2009) Stem cells and liver regeneration. Gastroenterology 137(2):466–481
Ellis AJ, Hughes RD, Nicholl D (1999) Temporary extracorporeal liver support for severe acute alcoholic hepatitis using BioLogic-DT. Int J Artif Organs 22:27–34
Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292(5819):154–156
Fang B, Shi M, Liao L et al (2004) Systemic infusion of FLK1(+) mesenchymal stem cells ameliorate carbon tetrachloride-induced liver fibrosis in mice. Transplantation 78:83–88
Fausto N (2004) Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. Hepatology 39:1477–1487
Fausto N, Campbell JS (2003) The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 120:117–130
Fausto N, Campbell JS, Riehle KJ (2006) Liver regeneration. Hepatology 43:S45–S53
Fellous TG, Islam S, Tadrous PJ, Elia G, Kocher HM, Bhattacharya S, Mears L, Turnbull DM, Taylor RW, Greaves LC, Chinnery PF, Taylor G, McDonald SA, Wright NA, Alison MR (2009) Locating the stem cell niche and tracing hepatocyte lineages in human liver. Hepatology 49(5):1655–1663
Fisher RA, Strom SC (2006) Human hepatocyte transplantation: worldwide results. Transplantation 82:441–449
Fitzpatrick E, Mitry RR, Dhawan A (2009) Human hepatocyte transplantation: state of the art. J Intern Med 266(4):339–357
Fox IJ, Chowdhury JR, Kaufman SS et al (1998) Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N Engl J Med 338:1422–1426
Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr et al (2002) Peginterferon alpha 2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975–982
Gaia S, Smedile A, Omede P et al (2006) Feasibility and safety of G-CSF administration to induce bone marrow-derived cells mobilization in patients with end stage liver disease. J Hepatol 45:13–19
Gao G, Lu Y, Calcedo R et al (2006) Biology of AAV serotype vectors in liver-directed gene transfer to nonhuman primates. Mol Ther 13:77–87
Garg V, Garg H, Khan A, Trehanpati N, Kumar A, Sharma BC, Sakhuja P, Sarin SK (2012) Granulocyte-colony stimulating factor mobilizes CD34+ cells and improves survival of patients with acute-on-chronic liver failure. Gastroenterology 142(3):505–512
Ghosh A, Allamarvdasht M, Pan CJ et al (2006a) Long-term correction of murine glycogen storage disease type Ia by recombinant adeno-associated virus-1-mediated gene transfer. Gene Ther 13:321–329
Ghosh SS, Gopinath P, Ramesh A (2006b) Adenoviral vectors: a promising tool for gene therapy. Appl Biochem Biotechnol 133:9–29
Gordon MY, Levicar N, Pai M et al (2006) Characterisation and clinical application of human CD34+ stem/progenitor cell populations mobilized into the blood by granulocyte colony-stimulating factor. Stem Cells 24:1822–1830
Gorla GR, Malhi H, Gupta S (2001) Polyploidy associated with oxidative injury attenuates proliferative potential of cells. J Cell Sci 114(Pt 16):2943–2951
Gouon-Evans V, Boussemart L, Gadue P, Nierhoff D, Koehler CI, Kubo A, Shafritz DA, Keller G (2006) BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. Nat Biotechnol 24:1402–1411
Grieger JC, Samulski RJ (2005) Adeno-associated virus as a gene therapy vector: vector development, production and clinical applications. Adv Biochem Eng Biotechnol 99:119–145

Grompe M, Overturf K, Al-Dhalimy M, Finegold M (1998) Therapeutic trials in the murine model of hereditary tyrosinaemia type I: a progress report. J Inherit Metab Dis 21:518–531
Habibullah CM, Syed IH, Qamar A, Taher-Uz Z (1994) Human fetal hepatocyte transplantation in patients with fulminant hepatic failure. Transplantation 58(8):951–952
Haridass D, Yuan Q, Becker PD, Cantz T, Iken M, Rothe M, Narain N, Bock M, Nörder M, Legrand N, Wedemeyer H, Weijer K, Spits H, Manns MP, Cai J, Deng H, Di Santo JP, Guzman CA, Ott M (2009) Repopulation efficiencies of adult hepatocytes, fetal liver progenitor cells, and embryonic stem cell-derived hepatic cells in albumin-promoter-enhancer urokinase-type plasminogen activator mice. Am J Pathol 175(4):1483–1492
Heo J et al (2006) Hepatic precursors derived from murine embryonic stem cells contribute to regeneration of injured liver. Hepatol 44(6):1478–1486
Herzog RW, Yang EY, Couto LB et al (1999) Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. Nat Med 5:56–63
Herzog EL, Chai L, Krause DS (2003) Plasticity of marrow-derived stem cells. Blood 102: 3483–3493
Horslen SP, McCowan TC, Goertzen TC et al (2003) Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. Pediatrics 111:1262–1267
Houlihan DD, Newsome PN (2008) Critical review of clinical trials of bone marrow stem cells in liver disease. Gastroenterology 135:438–450
Howden SE, Voullaire L, Vadolas J (2007) The transient expression of mRNA coding for Rep protein from AAV facilitates targeted plasmid integration. J Gene Med 10:42–50
Huang P, He Z, Ji S, Sun H, Xiang D, Liu C, Hu Y, Wang X, Hui L (2011) Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 475(7356):386–389
Hughes RD, Mitry RR, Dhawan A (2005) Hepatocyte transplantation for metabolic liver disease: UK experience. J R Soc Med 98:341–345
Jager L, Ehrhardt A (2007) Emerging adenoviral vectors for stable correction of genetic disorders. Curr Gene Ther 7:272–283
Jalan R, Wright G, Davies NA, Hodges SJ (2007) L-Ornithine phenylacetate (OP): a novel treatment for hyperammonemia and hepatic encephalopathy. Med Hypotheses 69:1064–1069
Jungermann K, Kietzmann T (2000) Oxygen: modulator of metabolic zonation and disease of the liver. Hepatology 31(2):255–260
Kafri T, Blomer U, Peterson DA, Gage FH, Verma IM (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat Genet 17:314–317
Kanazawa Y, Verma IM (2003) Little evidence of bone marrow-derived hepatocytes in the replacement of injured liver. Proc Natl Acad Sci USA 100(Suppl 1):11850–11853
Khan AA, Parveen N, Mahaboob VS, Rajendraprasad A, Ravindraprakash HR, Venkateswarlu J, Rao P, Pande G, Narusu ML, Khaja MN, Pramila R, Habeeb A, Habibullah CM (2008) Treatment of Crigler-Najjar Syndrome type 1 by hepatic progenitor cell transplantation: a simple procedure for management of hyperbilirubinemia. Transplant Proc 40(4):1148–1150
Khuroo MS, Khuroo MS, Farahat KL (2004) Molecular adsorbent recirculating system for acute and acute-on-chronic liver failure: a meta-analysis. Liver Transpl 10:1099–1106
Kim IH, Jozkowicz A, Piedra PA, Oka K, Chan L (2001) Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. Proc Natl Acad Sci USA 98:13282–13287
Kisseleva T, Uchinami H, Feirt N et al (2006) Bone marrow-derived fibrocytes participate in pathogenesis of liver fibrosis. J Hepatol 45:429–438
Kobayashi N, Ito M, Nakamura J et al (2000) Hepatocyte transplantation in rats with decompensated cirrhosis. Hepatology 31:851–857
Kobayashi H, Carbonaro D, Pepper K et al (2005) Neonatal gene therapy of MPS I mice by intravenous injection of a lentiviral vector. Mol Ther 11:776–789
Kramer L, Gendo A, Funk G, Madi C, Falkenhagen D, Gangl A (2000) Clinical experience with artifical liver support in chronic liver failure with encephalopathy. ASAIO J 46(2):a211

Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M (2000) Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 6:1229–1234

Levicar N, Pai M, Habib NA et al (2008) Long-term clinical results of autologous infusion of mobilized adult bone marrow derived CD34_ cells in patients with chronic liver disease. Cell Prolif 41:115–125

Li W, Wang D, Qin J, Liu C, Zhang Q, Zhang X, Yu X, Lahn BT, Mao FF, Xiang AP (2010) Generation of functional hepatocytes from mouse induced pluripotent stem cells. J Cell Physiol 222:492–501

Lyra AC, Soares MB, da Silva LF et al (2007a) A pilot randomised controlled study usd to evaluate efficacy of autologous bone marrow mononuclear cells transplantation in patients with advanced chronic liver disease (abstr). Hepatology 46(Suppl 1):271A

Lyra AC, Soares MB, da Silva LF et al (2007b) Feasibility and safety of autologous bone marrow mononuclear cell transplantation in patients with advanced chronic liver disease. World J Gastroenterol 13:1067–1073

Manno CS, Pierce GF, Arruda VR et al (2006) Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med 12:342–347

Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD (2001) Pegylated interferon alpha-2b plus ribavirin compared with interferon alpha-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet 358:958–965

Marshall E (1999) Gene therapy death prompts review of adenovirus vector. Science 286:2244–2245

Marshall A, Rushbrook S, Davies SE, Morris LS, Scott IS, Vowler SL, Coleman N, Alexander G (2005) Relation between hepatocyte G1 arrest, impaired hepatic regeneration, and fibrosis in chronic hepatitis C virus infection. Gastroenterology 128:33–42

Mazariegos GV, Kramer DJ, Lopez RC (2001) Safety observations in phase I clinical evaluation of the Excorp Medical bioartificial liver support system after the first four patients. ASAIO J 47:471–475

McCuskey R (1994) The hepatic microvascular system. In: Arias IM et al (eds) The liver: biology and pathobiology, 3rd edn., pp 1089–1106, New York, Raven Press

Merle U, Encke J, Tuma S, Volkmann M, Naldini L, Stremmel W (2006) Lentiviral gene transfer ameliorates disease progression in Long-Evans cinnamon rats: an animal model for Wilson disease. Scand J Gastroenterol 41:974–982

Meyburg J, Das AM, Hoerster F, Lindner M, Kriegbaum H, Engelmann G, Schmidt J, Ott M, Pettenazzo A, Luecke T, Bertram H, Hoffmann GF, Burlina A (2009) One liver for four children: first clinical series of liver cell transplantation for severe neonatal urea cycle defects. Transplantation 87(5):636–641

Miao CH, Thompson AR, Loeb K, Ye X (2001) Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer in vivo. Mol Ther 3:947–957

Michalopoulos GK, DeFrances MC (1997) Liver regeneration. Science 276:60–66

Mingozzi F, High KA (2007) Immune responses to AAV in clinical trials. Curr Gene Ther 7:316–324

Mishra L, Banker T, Murray J, Byers S, Thenappan A, He AR, Shetty K, Johnson L, Reddy EP (2009) Liver stem cells and hepatocellular carcinoma. Hepatology 49(1):318–329

Mitry RR, Dhawan A, Hughes RD, Bansal S, Lehec S, Terry C et al (2004) One liver, three recipients: segment IV from split-liver procedures as a source of hepatocytes for cell transplantation. Transplantation 77:1614–1616

Mitzner SR, Stange J, Klammt S, Koball S, Hickstein H, Reisinger EC (2009) Albumin dialysis MARS: knowledge from 10 years of clinical investigation. ASAIO J 55(5):498–502

Miyazawa K et al (1989) Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. Biochem Biophys Res Commun 163:967–973

Mohamadnejad M, Alimoghaddam K, Mohyeddin-Bonab M et al (2007a) Phase 1 trial of autologous bone marrow mesenchymal stem cell transplantation in patients with decompensated liver cirrhosis. Arch Iran Med 10:459–466

Mohamadnejad M, Namiri M, Bagheri M et al (2007b) Phase 1 human trial of autologous bone marrow-hematopoietic stem cell transplantation in patients with decompensated cirrhosis. World J Gastroenterol 28:3359–3363

Muir AJ, Sylvestre PB, Rockey DC (2006) Interferon gamma-1b for the treatment of fibrosis in chronic hepatitis C infection. J Viral Hepat 13(5):322–328

Muraca M, Gerunda G, Neri D, Vilei MT, Granato A, Feltracco P et al (2002) Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. Lancet 359:317–318

Nakamura T, Nawa K, Ichihara A (1984) Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem Biophys Res Commun 122:1450–1459

Nakamura T et al (1989) Molecular cloning and expression of human hepatocyte growth factor. Nature 342:440–443

Nakamura T, Torimura T, Sakamoto M et al (2007) Significance and therapeutic potential of endothelial progenitor cell transplantation in a cirrhotic liver rat model. Gastroenterology 133:91–107

Newsome PN, Johannessen I, Boyle S, Dalakas E, McAulay KA, Samuel K, Rae F, Forrester L, Turner ML, Hayes PC, Harrison DJ, Bickmore WA, Plevris JN (2003) Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. Gastroenterology 124:1891–1900

Regulation (EC) No 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending directive 2001/83/EC and regulation (EC) No 726/2004. Official Journal of the European Union (10.12. 2007) L324/121

Nussler A, Konig S, Ott M et al (2006) Present status and perspectives of cell-based therapies for liver diseases. J Hepatol 45:144–159

Ohashi K, Yokoyama T, Yamato M, Kuge H, Kanehiro H, Tsutsumi M, Amanuma T, Iwata H, Yang J, Okano T, Nakajima Y (2007) Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. Nat Med 13(7):880–885

Ohashi K, Koyama F, Tatsumi K, Shima M, Park F, Nakajima Y, Okano T (2010) Functional lifelong maintenance of engineered liver tissue in mice following transplantation under the kidney capsule. J Tissue Eng Regen Med 4(2):141–148

Oka K, Belalcazar LM, Dieker C et al (2007) Sustained phenotypic correction in a mouse model of hypoalphalipoproteinemia with a helper-dependent adenovirus vector. Gene Ther 14:191–202

Overturf K, Al Dhalimy M, Tanguay R et al (1996) Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I. Nat Genet 12:266–273

Overturf K, A-Dhalimy M, Ou CN et al (1997a) Adenovirus-mediated gene therapy in a mouse model of hereditary tyrosinemia type I. Hum Gene Ther 8:513–521

Overturf K, al-Dhalimy M, Ou CN, Finegold M, Grompe M (1997b) Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes. Am J Pathol 151:1273–1280

Oyagi S, Hirose M, Kojima M et al (2006) Therapeutic effect of transplanting HGF-treated bone marrow mesenchymal cells into CCl4- injured rats. J Hepatol 44:742–748

Pai M, Zacharoulis D, Milicevic MN, Helmy S, Jiao LR et al (2008) Autologous infusion of expanded mobilized adult bone marrow-derived CD34+ cells into patients with alcoholic liver cirrhosis. Am J Gastroenterol 103:1952–1958

Peng Z (2005) Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. Hum Gene Ther 16:1016–1027

Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP (1999) Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170

Pockros PJ, Jeffers L, Afdhal N, Goodman ZD, Nelson D et al (2007) Final results of a double-blind, placebo-controlled trial of the antifibrotic efficacy of interferon-gamma1b in chronic hepatitis C patients with advanced fibrosis or cirrhosis. Hepatology 45(3):569–578

Ponder KP, Melniczek JR, Xu L et al (2002) Therapeutic neonatal hepatic gene therapy in mucopolysaccharidosis VII dogs. Proc Natl Acad Sci USA 99:13102–13107

Poyck PP, van Wijk AC, van der Hoeven TV, de Waart DR, Chamuleau RA, van Gulik TM, Oude Elferink RP, Hoekstra R (2008) Evaluation of a new immortalized human fetal liver cell line (cBAL111) for application in bioartificial liver. J Hepatol 48(2):266–275
Quante M, Wang TC (2009) Stem cells in gastroenterology and hepatology. Nat Rev Gastroenterol Hepatol 6(12):724–737
Quintana-Bustamante O, Alvarez-Barrientos A, Kofman AV, Fabregat I, Bueren JA, Theise ND, Segovia JC (2006) Hematopoietic mobilization in mice increases the presence of bone marrow-derived hepatocytes via in vivo cell fusion. Hepatology 43:108–116
Raper SE, Wilson JM, Yudkoff M, Robinson MB, Ye X, Batshaw ML (1998) Developing adenoviral-mediated in vivo gene therapy for ornithine transcarbamylase deficiency. J Inherit Metab Dis 21(Suppl 1):119–137
Rathjen J, Rathjen PD (2001) Mouse ES cells: experimental exploitation of pluripotent differentiation potential. Curr Opin Genet Dev 11:587–594
Rettinger SD, Ponder KP, Saylors RL, Kennedy SC, Hafenrichter DG, Flye MW (1993) In vivo hepatocyte transduction with retrovirus during in-flow occlusion. J Surg Res 54:418–425
Rifai K, Ernst T, Kretschmer U (2003) Prometheus: a new extracorporeal system for the treatment of liver failure. J Hepatol 39:984–990
Rifai K, Kribben A, Gerken G et al (2010) Extracorporeal liver support by fractionated plasma separation and absorption (Prometheus®) in patients with acute on chronic liver failure (HELIOS study): a prospective randomized controlled multicenter study. J Hepatol 52(Suppl 1):S3
Roskams T (2006) Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. Oncogene 25(27):3818–3822
Roskams T, Yang SQ, Koteish A, Durnez A, DeVos R, Huang X, Achten R, Verslype C, Diehl AM (2003) Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. Am J Pathol 163:1301–1311
Russell WE, Mcgowan JA, Bucher NL (1984) Partial characterization of a hepatocyte growth factor from platelets. J Cell Physiol 119:183–192
Russo FP, Alison MR, Bigger BW et al (2006) The bone marrow functionally contributes to liver fibrosis. Gastroenterology 130:1807–1821
Sabatino DE, Mackenzie TC, Peranteau W et al (2007) Persistent expression of hF.IX after tolerance induction by in utero or neonatal administration of AAV-1-F.IX in hemophilia B mice. Mol Ther 15:1677–1685
Sakaida I, Terai S, Yamamoto N et al (2004) Transplantation of bone marrow cells reduces CCL4-induced liver fibrosis in mice. Hepatology 40:1304–1311
Saliba F, Camus C, Durand F, Mathurin P, Delafosse B, Barange K, Perrigault P-F, Revel P, Serfaty L, Belnard M, Letierce A, Ichai P, Samuel D (2009) Predictive factors of transplant free survival in patients with fulminant and subfulminant hepatic failure: results from a randomized controlled multicenter trial. J Hepatol 50(suppl 1):S89–S90
Samulski RJ, Zhu X, Xiao X et al (1991) Targeted integration of adeno-associated virus (AAV) into human chromosome 19. EMBO J 10:3941–3950
Santoro A, Faenza S, Mancini E (2006) Prometheus system: a technological support in liver failure. Transplant Proc 38:1078–1082
Sauer IM, Kardassis D, Zeillinger K (2003) Clinical extracorporeal hybrid liver support: phase I study with primary porcine liver cells. Xenotransplantation 10:460–469
Schiff ER, Sorrell MF, Maddrey WC (2007) Schiff's diseases of the liver. Lippincott Williams & Wilkins, Philadelphia
Schmidt C et al (1995) Scatter factor/hepatocyte growth factor is essential for liver development. Nature 373:699–702
Schneider et al (2006) 15 Hepatocyte transplantation in an acute liver failure due to mushroom poisoning. Transplantation 82(8):1115–1116
Sekiya S, Suzuki A (2011) Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 475(7356):390–393

Seppen J, van der Rijt R, Looije N, van Til NP, Lamers WH, Oude Elferink RP (2003) Long-term correction of bilirubin UDP glucuronyltransferase deficiency in rats by in utero lentiviral gene transfer. Mol Ther 8:593–599

Sherlock S, Dooley J (2002) Disease of the liver and biliary system. Blackwell Publishing Company, Oxford

Sigal SH, Gupta S, Gebhard DF Jr, Holst P, Neufeld D, Reid LM (1995) Evidence for a terminal differentiation process in the rat liver. Differentiation 59:35–42

Siller-Lopez F, Sandoval A, Salgado S, Salazar A, Bueno M (2004) Treatment with human metalloproteinase-8 gene delivery ameliorates experimental rat liver cirrhosis. Gastroenterology 126:1122–1133

Sinn PL, Sauter SL, McCray PB Jr (2005) Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors – design, biosafety, and production. Gene Ther 12:1089–1098

Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA (2010) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51:297–305

Sokal EM, Smets F, Bourgois A, Van Maldergem L, Buts JP, Reding R et al (2003) Hepatocyte transplantation in a 4-year-old girl with peroxisomal biogenesis disease: technique, safety, and metabolic follow-up. Transplantation 76:735–738

Song Z, Cai J, Liu Y, Zhao D, Yong J, Duo S, Song X, Guo Y, Zhao Y, Qin H, Yin X, Wu C, Che J, Lu S, Ding M, Deng H (2009) Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. Cell Res 19:1233–1242

Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K (2008) Induced pluripotent stem cells generated without viral integration. Science 322:945–949

Stange J, Mitzner S, Risler T (1999) Molecular adsorbent recycling system (MARS): clinical results of a new membrane-based blood purification system for bioartificial liver support. Artif Organs 23:319–330

Steiner C, Sen S, Stange J, Williams R, Jalan R (2004) Binding of bilirubin and bromosulphthalein to albumin: implications for understanding the pathophysiology of liver failure and its management. Liver Transpl 10:1531–1538

Stephenne X, Najimi M, Smets F, Reding R, de Ville de Goyet J, Sokal EM (2005) Cryopreserved liver cell transplantation controls ornithine transcarbamylase deficient patient while awaiting liver transplantation. Am J Transplant 5:2058–2061

Stephenne X, Najimi M, Sibille C, Nassogne MC, Smets F, Sokal EM (2006) Sustained engraftment and tissue enzyme activity after liver cell transplantation for argininosuccinate lyase deficiency. Gastroenterology 130:1317–1323

Stutchfield BM, Rashid S, Forbes SJ, Wigmore SJ (2010) Practical barriers to delivering autologous bone marrow stem cell therapy as an adjunct to liver resection. Stem Cells Dev 19(2):155–161

Sullivan GJ, Hay DC, Park IH, Fletcher J, Hannoun Z, Payne CM, Dalgetty D, Black JR, Ross JA, Samuel K, Wang G, Daley GQ, Lee JH, Church GM, Forbes SJ, Iredale JP, Wilmut I (2010) Generation of functional human hepatic endoderm from human induced pluripotent stem cells. Hepatology 51:329–335

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872

Takezawa R, Watanabe Y, Akaike T (1995) Direct evidence of macrophage differentiation from bone marrow cells in the liver: a possible origin of Kupffer cells. J Biochem 118:1175–1183

Taniguchi E, Kin M, Torimura T et al (2006) Endothelial progenitor cell transplantation improves the survival following liver injury in mice. Gastroenterology 130:521–531

Taub R (1996) Liver regeneration 4: transcriptional control of liver regeneration. FASEB J 10(4):413–427

Taub R (2004) Liver regeneration: from myth to mechanism. Nat Rev Mol Cell Biol 5(10):836–847

Terai S, Ishikawa T, Omori K et al (2006) Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. Stem Cells 24:2292–2298
The Journal of Gene Medicine Clinical Trial site [Cited 22/12/2008] [updated Jun 2012]
Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, Krause DS (2000) Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 31:235–240
Thieringer F, Maass T, Czochra P, Klopcic B, Conrad I et al (2008) Spontaneous hepatic fibrosis in transgenic mice overexpressing PDGF-A. Gene 423(1):23–28
Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282(5391):1145–1147
Thorgeirsson SS (1996) Hepatic stem cells in liver regeneration. FASEB J 10:1249–1256
Tominaga K, Kuriyama S, Yoshiji H et al (2004) Repeated adenoviral administration into the biliary tract can induce repeated expression of the original gene construct in rat livers without immunosuppressive strategies. Gut 53:1167–1173
Touboul T, Hannan NR, Corbineau S, Martinez A, Martinet C, Branchereau S, Mainot S, Strick-Marchand H, Pedersen R, Di Santo J, Weber A, Vallier L (2010) Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. Hepatology 51(5):1754–1765
Transplant Proc 2008 40(4, May):1148–50
Ueki T, Kaneda Y, Tsutsui H, Nakanishi K, Sawa Y et al (1999) Hepatocyte growth factor gene therapy of liver cirrhosis in rats. Nat Med 5(2):226–230
Ueno T, Nakamura T, Torimura T et al (2006) Angiogenic cell therapy for hepatic fibrosis. Med Mol Morphol 39:16–21
Van De Kerkhove MP, Di Florio E, Scuderi V (2002) Phase I clinical trial with the AMC-bioartificial liver. Int J Artif Organs 25:950
Vassilopoulos G, Wang PR, Russell DW (2003) Transplanted bone marrow regenerates liver by cell fusion. Nature 422:901–904
Waddington SN, Nivsarkar MS, Mistry AR et al (2004) Permanent phenotypic correction of hemophilia B in immunocompetent mice by prenatal gene therapy. Blood 104:2714–2721
Wagers AJ, Sherwood RI, Christensen JL, Weissman IL (2002) Little evidence for developmental plasticity of adult hematopoietic stem cells. Science 297:2256–2259
Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M (2003) Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 422:897–901
Wiemann SU, Satyanarayana A, Tsahuridu M, Tillmann HL, Zender L, Klempnauer J, Flemming P, Franco S, Blasco MA, Manns MP, Rudolph KL (2002) Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. FASEB J 16:935–942
Willenbring H, Bailey AS, Foster M, Akkari Y, Dorrell C, Olson S, Finegold M, Fleming WH, Grompe M (2004) Myelomonocytic cells are sufficient for therapeutic cell fusion in liver. Nat Med 10:744–748
Wu Z, Asokan A, Samulski RJ (2006) Adeno-associated virus serotypes: vector toolkit for human gene therapy. Mol Ther 14:316–327
Yannaki E, Anagnostopoulos A, Kapetanos D et al (2006) Lasting amelioration in the clinical course of decompensated alcoholic cirrhosis with boost infusions of mobilized peripheral blood stem cells. Exp Hematol 34:1583–1587
Ye P, Thompson AR, Sarkar R et al (2004) Naked DNA transfer of Factor VIII induced transgene-specific, species-independent immune response in hemophilia A mice. Mol Ther 10:117–126
Yiu WH, Pan CJ, Allamarvdasht M, Kim SY, Chou JY (2007) Glucose-6-phosphate transporter gene therapy corrects metabolic and myeloid abnormalities in glycogen storage disease type Ib mice. Gene Ther 14:219–226
Young SM Jr, McCarty DM, Degtyareva N, Samulski RJ (2000) Roles of adeno-associated virus Rep protein and human chromosome 19 in site-specific recombination. J Virol 74:3953–3966
Zhao DC, Lei JX, Chen R et al (2005) Bone marrow derived mesenchymal stem cells protect against experimental liver fibrosis in rats. World J Gastroenterol 11:3431–3440

Chapter 38
Kidney

Sajoscha A. Sorrentino and Hermann Haller

Abstract Regenerative medicine is an area of intense excitement and potential. Despite the increasing rate of end-stage renal disease, dialysis and transplantation remain the only treatment options to date. However, there is hope that stem cells and regenerative medicine may procure additional therapeutic options for renal disease. Such new treatment options may include induction of repair using endogenous or exogenous stem cells or the reprogramming of the kidney to reinitiate development. This chapter reviews the current state of understanding with respect to stem cell functions in the kidney, regenerative principles in kidney diseases, as well as clinical implications and implementation of regenerative medicine in renal disease.

38.1 Introduction

The term *regenerative medicine* spans bioengineering, cell biology and matrix biology with the objective to repair or re-grow a damaged organ or tissue. It can be defined as the use of cells for the treatment of a disease and covers both organ repair and the *de novo* regeneration of an entire organ. Organ repair can be delivered *in situ* or *ex vivo*. *In situ* possibilities include the recruitment of stem cells to the kidney to trigger repair and the induction of dedifferentiation of resident renal cells. Whereas some regard *in situ* approaches as more likely to be successful for an architecturally and anatomically constrained organ such as the kidney, the other approach is the

S.A. Sorrentino, M.D.
Department of Radiology, Hannover Medical School,
Carl-Neuberg-Str. 1, 30625 Hannover, Germany

H. Haller, M.D. (✉)
Department of Nephrology and Hypertension, Hannover Medical School,
Carl-Neuberg-Str. 1, 30625 Hannover, Germany
e-mail: Haller.Hermann@mh-hannover.de

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_38,

ex vivo culture of stem cells for re-delivery to the damaged kidney. This might involve autologous or non-autologous stem cells from a variety of sources. Finally, a bioengineering approach that relies on cells, factors, and matrix may be achievable. Although seemingly the most difficult, it may be the more feasible approach for genetic conditions such as polycystic kidney disease (Little 2006).

38.2 Kidney Development, Stem Cell Function

Regenerative biology draws on an understanding of normal developmental processes. Understanding the molecular basis of kidney development will be the key to the development of regenerative therapies for chronic renal disease (Little (Horster et al. 1999). During mammalian development, three separate excretory organs develop: (Horster et al. (Horster et al. 1999). The pronephros, the mesonephros, and the metanephros. In mammals, it is the paired metanephroi that persist postnatally and constitute the permanent kidney. The permanent kidney arises via reciprocal interactions between two tissues, the ureteric bud and the metanephric mesenchyme, the latter arising from the intermediate mesoderm. The ureteric bud gives rise to the collecting ducts and the ureter. The metanephric mesenchyme, which shows much broader potential and gives rise to all other elements of the nephrons, the interstitium, and the vasculature, is regarded as the renal progenitor population (Herzlinger et al. 1992). As the ureteric bud reaches the metanephric mesenchyme, signals from the tips of the branching UB induce areas of adjacent metanephric mesenchyme to aggregate and undergo a mesenchyme-to-epithelial (MET) transition. Each MET event represents the birth of a new nephron with the first nephrons "born" in the center of the metanephric mesenchyme. The peripheral metanephric mesenchyme, which has not yet undergone induction, is referred to as the nephrogenic zone. Nephrogenesis in humans is complete by week 36 of gestation, whereas it continues for 1–2 weeks after birth in the mouse and the rat. At that time, it is assumed that the peripheral nephrogenic zone is exhausted (Horster et al. 1999).

Embryonic metanephroi, differentiating into the adult kidney, have come to be a generally accepted model system for organogenesis. Nephrogenesis implies a highly controlled series of morphogenetic and differentiation events that starts with reciprocal inductive interactions between two different primordial tissues and leads, in one of two mainstream processes, to the formation of mesenchymal condensations and aggregates. These go through the intricate process of mesenchyme-to-epithelium transition by which epithelial cell polarization is initiated, and they continue to differentiate into the highly specialized epithelial cell populations of the nephron. Each step along the developmental metanephrogenic pathway is initiated and organized by signaling molecules that are locally secreted polypeptides encoded by different gene families and regulated by transcription factors. Nephrogenesis proceeds from the deep to the outer cortex, and it is directed by a second, entirely different developmental process, the ductal branching of the ureteric bud-derived collecting tubule. Both systems, the nephrogenic (mesenchymal) and the ductogenic (ureteric), undergo a repeat series

of inductive signaling that serves to organize the architecture and differentiated cell functions in a cascade of developmental gene programs(Horster et al. 1999).

The development of the metanephric (permanent) mammalian kidney begins at gestational *week 4–5* in humans and at E11 in mouse. Organogenesis and its governing principles have been studied mostly in the mouse. Metanephros formation, i.e., organogenesis of the permanent kidney, is initiated by the ureteric bud, which sprouts out of the posterior end of the Wolffian duct and invades the metanephrogenic mesenchyme. The subsequent interaction between the two primordia induces the ureteric bud to branch dichotomously, thus initiating the morphogenesis of the collecting duct system. Induced metanephric mesenchyme condenses at the tips of the ureteric buds, and mesenchymal cells form aggregates, thus beginning the mesenchyme-to-epithelium transition. Each aggregate epithelializes (Sariola et al. 1983) and proceeds in stages to the vesicle stage, comma stage, and S-stage, from where each S-shaped body, after fusion with the ureteric bud-derived collecting duct, differentiates into one of the (2×10^6) nephrons of the human kidneys. The architectural pattern, therefore, as a result of the sequential ureteric bud arborization, is designed to proceed from the deep cortex to the periphery in a repeat series of induction, morphogenesis, and differentiation (Horster et al. 1999).

The epithelial segments of the nephron, unlike the ureteric bud-derived collecting duct system, are created from mesenchymal cells by an intricate cascade of events. The early events result in the acquisition of an essentially epithelial character by the future nephron cells while these polarized cells form a sphere or vesicle. The process of modeling the subsequent stages of comma and S-shape is not yet fully understood, although plenty of morphoregulatory molecules and transcription factors are sequentially and differentially expressed. These stages of morphogenesis are the onset of nephron differentiation, i.e., epithelial segments begin to express their specific properties (Horster 1985). These stages of nephrogenesis have an ancestry that begins at the blastula stage, which determines the mesoderm; it follows the induction of the pronephros and the directed migration of the pronephric duct to proceed through the stage of the Wolffian duct and to induce the metanephric mesenchyme, which in turn directs branching of the ureteric tree. Cells of the metanephrogenic mesenchyme are induced by ureteric bud cells to become stem cells after rescue from apoptosis; they go on to condense and, guided by regulatory circuits of gene expression and repression, to enter the mesenchyme-to-epithelium transition, and to polarize to apicobasal expression patterns (Horster et al. 1999). For the metanephric mesenchymal blastema to produce the ~15 epithelial cell types of the metanephric kidney, it must be induced to undergo a conversion to the epithelial phenotype and subsequently differentiate into the highly specialized cell types of the nephron. Hypothetically, this pathway could start from two different points. One starting point would be a homogeneous mesenchymal population consisting of one multipotent cell type from which all nephron epithelial cell types are derived. Alternatively, the primary inductive event is not the conversion to the epithelial phenotype but a commitment of the mesenchymal cell type to different developmental pathways, and the secondary inductive event of phenotypic conversion then destines already committed cells to

be recruited for the early nephron (Koseki et al. 1991; Herzlinger et al. 1992; Qiao et al. 1995). Studies on the temporospatial expression of two transcription factors: *BF-2* (Hatini et al. 1996) and *Pax-2* (Dressler et al. 1990; Dressler 1997), have shed some light on this situation. It seems now justified to favor the hypothesis that all peripheral mesenchymal blastema cell types are induced to become stem cells through the first signal interactions. This initial step rescues most of the nephrogenic stem cells now expressing *Pax-2* from apoptosis (Koseki et al. 1992; Barasch et al. 1996), whereas the uninduced mesenchymal cells enter programmed cell death. Induction is a two-step event (Barasch et al. 1996) that had been postulated already from earlier tissue recombination work, (Saxen et al. 1983) where it was found that a short-time (hours) exposure of uninduced mesenchyme to the ureteric inductor led to the stem cell phenotype but no further. Nevertheless, this first step to the stem cell phenotype rescues most of the mesenchyme from apoptosis. The second step, however, very likely differs in molecular nature from the first one (Barasch et al. 1996). Two hypotheses, at present, are similarly supported by data, although not yet by complete lineage analysis. In one, the primary inductive interactions between mesenchyme and bud are believed to determine the distinct and final developmental pathways of both stromal and nephrogenic lineage (Ekblom 1989). In the other, a bivalent stem cell progenitor population that gathers next to the outermost ureteric bud cells is available throughout nephrogenesis, and it may either take the nephrogenic (Pax-2) or the stromogenic (BF-2) pathway (Saxen and Sariola 1987; Davies and Bard 1996; Hatini et al. 1996). It is interesting to note that the endothelial progenitor cell, the angioblast, may derive also from a bipotential (mesodermal) stem cell precursor (Risau 1997). Cell lineage analysis based on classic embryologic work (Herzlinger 1994) clearly indicates that the definitive kidney is derived from two independent tissue compartments of the intermediate mesoderm, namely, the metanephrogenic mesenchyme and the Wolffian duct. This traditional view has been broadened by a set of data derived from embryonic kidney organ culture (Qiao et al. 1995), when uninduced mesenchyme was isolated and tagged so that cells could be followed to their final destination, and then cocultured with isolated ureteric bud, mesenchymal cells were found to be inserted into the collecting duct, although the majority of collecting duct cells were derived from the ureteric bud. Organogenesis of the kidney has long become a model system that represents principles in morphogenesis and cell differentiation. The continuous process of morphogenesis is guided by cascades of interactions between two different cell populations. Regulation involves diverse families of genes and their products, including protooncogene-encoded receptors and their polypeptide ligands, transcription factors and their target genes, and regulating extracellular matrix proteins and CAM-mediated signals. All of these diverse systems interact to initiate and guide embryonic renal morphogenesis and cell differentiation (Horster et al. 1999). As distinct from other tissues, such as bone marrow, it has been difficult to isolate or confirm the existence of stem cells in the kidney, although several studies have suggested the existence of stem cells in the adult renal interstitium (Hishikawa and Fujita 2006).

38.2.1 Slow-Cycling Cells in the Papilla

Adult stem cells are considered to have a slow cycling time, (Cotsarelis et al. 1989; Johansson et al. 1999; Lavker and Sun 2000) and thus Oliver et al. tried to distinguish the cells in the kidney by measuring the retention of the nucleotide label bromodeoxyuridine (BrdU), which is incorporated into the DNA of cells during DNA synthesis (Oliver et al. 2004). BrdU was administered to 3-day-old rat and mice pups. At this age, because nephrogenesis in rodents continues after birth, many cells in the kidney were probably dividing and thus could incorporate BrdU. After a chase period of at least 2 months, during which the multiple cell divisions required for kidney growth would have diluted the BrdU content of most cells, incorporation of BrdU was analyzed in the kidney tissue. Oliver et al. (2004) found that only the interstitium of the renal papilla contained an abundant population of cells that retained a strong BrdU signal. They also found that the cells entered the cell cycle and the BrdU signal quickly disappeared from the papilla in transient real ischemia models. Moreover, the isolated renal papilla cells were multi-potent and displayed other characteristics of adult stem cells, and when they were injected directly into the renal cortex, the cells incorporated into the kidney parenchyma (Hishikawa and Fujita 2006).

38.2.2 Side Population Cells

In 1996, Goodell et al. reported a new method of obtaining an enriched population of hematopoietic stem cells from adult bone marrow in a single step by Hoechst 33342 staining and FACS sorting (Goodell et al. 1996). The isolated cells were called side population (SP) cells, and the SP phenotype can be used to purify a stem cell–rich fraction. The SP phenotype is determined by the BCRP1/ABCG2 gene, and enforced expression of BCRP1/ABCG2 prevents hematopoietic differentiation (Zhou et al. 2001). To determine core genes comprising a stem cell genetic program, several comprehensive microarray studies have been performed (Ivanova et al. 2002; Ramalho-Santos et al. 2002). However, the number of overlapping genes among the reports was limited, and BCRP1/ABCG2 was the only gene that was expressed in ES cells, hematopoietic stem cells and neurosphere cells (Easterday et al. 2003). These results suggest that SP cells may play a role as adult stem cells. In fact, skeletal muscle SP cells (Asakura et al. 2002) may differentiate into endothelial cells (Majka et al. 2003) and bone marrow–derived SP cells into cardiomyocytes, endothelial cells (Jackson et al. 2001) and osteoblast precursors (Olmsted-Davis et al. 2003). Recently, Hishikawa et al. found that kidney SP cells differentiated into multiple lineages in the presence of leukemia inhibitory factor *via* kidney-specific cadherin 16 (Hishikawa et al. 2005b). Moreover, the function of kidney SP cells was found to be regulated by basic helix–loop-helix transcription factor MyoR, and the cells resided in the interstitial spaces of the kidney (Hishikawa et al. 2005a; Hishikawa and Fujita 2006).

38.2.3 CD133⁺ Cells in the Interstitium

CD133 is a surface marker of endothelial progenitor cells, hematopoietic progenitor cells and neural stem cells. Bussolati et al. reported that CD133-positive cells in the interstitium of the adult human kidney have characteristics of stem cells (Bussolati et al. 2005). The cells expressed the early nephron developmental marker Pax2, as well as several markers typical of bone marrow stromal cells, but were negative for hematopoietic cell markers such as CD34 or CD45. By using different culture conditions, the authors indicated that CD133+ renal cells might be pluripotent, having the capacity to differentiate into either type of tubular cells with the appropriate cues (Hishikawa and Fujita 2006).

38.2.4 rKS56 Cells

In the developing kidney, there are two major distinct areas of cell proliferation, the nephrogenic zone in the outer cortex below the renal capsule and the area in the corticomedullary junction corresponding to the primitive S3 segment of the proximal tubule (Cha et al. 2001). Kitamura et al. (2005) dissected individual nephrons from adult rat kidneys, then separated them into segments and cultured them (Hishikawa et al. 2005a, b). Outgrowing cells were replated after limiting dilution so that each well contained a single cell. In this way, they were able to isolate the cell line showing the most potent growth, which they designated rKS56. rKS56 cells expressed immature cell makers relating to kidney development and mature tubular markers. The location of rKS56 cells in kidney tissue is unclear, but rKS56 cells possessed self-renewal and multi-plasticity, and differentiated into mature tubular cells defined by aquaporin1,2 expression under different culture conditions. With the exception of rKS56 cells, all of the above types of cells were found in the interstitium but it is still unclear whether these cells are completely different types of cells or merely the same cells isolated by different methods. Moreover, the multipotency of these cells was evaluated under different conditions, and thus further studies will be required to conclusively determine the most potent cells that contribute to kidney regeneration or cell therapy (Hishikawa and Fujita 2006).

38.3 Kidney Diseases, Regenerative Principles

Most renal diseases can be envisioned as the consequence of a dysbalance between tissue damage and repair. Hypoxia, infection, immune reactions, and toxic substances can damage renal tissue (Rookmaaker et al. 2004). On the other hand, regenerative mechanisms counteracting the damage inflicted on renal tissues have been reported as well, both in tubuli (Toback 1992) and the glomeruli (Abouna et al. 1983). Insights

into the nature of these regenerative mechanisms have evolved over the years. In tissues with a high cell turnover like the intestine or the hematopoietic system, organ- or tissue-restricted stem cells have been shown to replace cells that have completed their life cycle. It is becoming increasingly apparent that in organs with a relatively low rate of cellular turnover like the liver and kidney similar regenerative mechanisms are operational (Pabst and Sterzel 1983 ; Imasawa et al. 2001 ; Poulsom et al. 2001). The cellular players responsible for regenerative mechanisms in the kidney include not only proliferating mature renal cells but recent reports also suggest a renal role for stem cells, both from local pools as well as from the circulation (Rookmaaker et al. 2004).

In the mammal, partial nephrectomy stimulates hypertrophy of remaining tissue, even in the contralateral kidney, but not the generation of new nephrons. However, whereas the resection of an adult kidney does not lead to the regeneration achieved in the liver, the mammalian kidney shares with the majority of organs the ability to repopulate and repair structures that have sustained some degree of injury. This process, termed *cellular repair*, can be achieved by reentry into mitosis and proliferation of neighboring cells. As a result, the kidney can undergo significant remodeling in response to acute damage. For example, obstruction of the ureter can result in the near destruction of the kidney medulla, but once the obstruction is removed, there is a rapid process of reconstruction and repair that will regenerate the tubules of the medulla without forming new nephrons. It has been proposed that the cells that elicit such repair come from interstitial cell transdifferentiation, tubular cell dedifferentiation and migration into the areas of damage before redifferentiation, the recruitment of stem cells from the bone marrow, or the generation of new tubular cells from an endogenous renal stem cell population (reviewed in reference [Davies and Bard 1996]). Which of these is primarily responsible for the *cellular repair* that is observed after acute damage, has not been proved definitively using lineage tracing. However, the mammalian kidney seems to have a very limited potential for *structural repair* or true regeneration. While nephrogenesis is occurring in the fetus, there is evidence that a systemic humoral response to nephrectomy allows the enhanced nephrogenesis of the remaining organ. However, nephrogenesis in mammals ceases just before or shortly after birth, and the birth of new nephrons has never been reported after this point in time. Chronic injury of the kidney, which is responsible for the majority of cases of end-stage renal failure, results in irreversible glomerular and tubular damage and resultant loss of renal function. Hence, mammalian kidneys respond to chronic damage by fibrosis, scarring, and irreversible functional loss.

38.3.1 Regeneration of Renal Endothelium

A number of studies have addressed maintenance and regeneration of the specialized renal glomerular capillaries. In normal rats, the rate of total glomerular cell renewal is about 1% per day with the endothelial fraction being the most predominant

cell type (Pabst and Sterzel 1983). However, in response to injury, the rate of vascular regeneration could well be increased. An established model to study glomerular injury and repair in rats is experimental anti-Thy1.1 glomerulonephritis. Injection of a complement-fixing antibody to the mesangial cell antigen Thy1.1 causes acute mesangiolysis and matrix dissolution, leading to ballooning of glomerular capillaries, formation of aneurysms, and loss of endothelial cells. In the subsequent repair phase increased proliferation and migration of endothelial and mesangial cells is observed resulting in (partial) restoration of glomerular structure and function (Rookmaaker et al. 2004). Using this model it was shown that glomerular capillary repair is associated with a marked increase in endothelial cell proliferation (Iruela-Arispe et al. 1995). Several studies have provided evidence that circulating endothelial progenitor cells (EPC) may contribute to glomerular capillary repair. Experiments by Rookmaaker et al. with rat hematopoietic chimeras demonstrated low levels of bone marrow–derived cells staining for the rat endothelial cell antigen RECA-1 (Rookmaaker et al. 2003). The number of these cells gradually increased over time suggesting that EPC contribute to normal physiologic glomerular endothelial cell turnover. Following anti-Thy- 1.1–induced glomerular injury they observed a 4x increase in bone marrow-derived endothelial cells in the glomeruli. These data indicate that glomerular repair cannot only be attributed to migration and proliferation of resident endothelial cells but also involves bone marrow-derived cells (Rookmaaker et al. 2004). Participation of circulating EPC to renal regeneration has also been demonstrated in human adults. As early as 1969 the presence of acceptor endothelial cells in kidney allografts was first reported (Williams and Alvarez 1969). It has been reported that in human renal transplants the extent of replacement of donor endothelial cells lining the peritubular capillaries by those of the acceptor was related to the severity of vascular injury (Lagaaij et al. 2001). It was suggested that this endothelial replacement could be explained by the involvement of acceptor-derived EPC. Rookmaaker et al. (2002) demonstrated male, donor-derived endothelial cells in the renal macrovasculature of a female patient who developed thrombotic microangiopathy after gender-mismatched bone marrow transplantation (Rookmaaker et al. 2002). Taken together these observations confirm a novel role for bone marrow–derived endothelial cells in maintenance and repair of renal endothelium.

38.3.2 Regeneration of the Renal Mesangium

Glomerular mesangial cells provide structural capillary support to the glomerulus and display a smooth muscle cell–like phenotype. They play a central role in the pathogenesis of a number of human and experimental glomerular inflammatory diseases. In particular, mesangial hyperplasia is a prominent histopathologic feature associated with impaired glomerular function. Although transient hyperplasia is thought to reflect a physiologic response required for successful glomerular

reconstitution and renal tissue repair, tight regulation of mesangial proliferation, function, and apoptosis is needed for recovery without fibrosis. Initially, mesangial maintenance and repair after injury was thought to depend solely on proliferation of viable resident intraglomerular mesangial cells (Rookmaaker et al. 2004). These mature mesangial cells dedifferentiate before they proliferate (El-Nahas 2003). Like the glomerular endothelial cells, in normal rats, mesangial cell turnover amounts to less than 1% per day (Pabst and Sterzel 1983). Hugo et al. demonstrated that during recovery of anti-Thy1.1 glomerulonephritis, proliferating immature mesangial cells migrated from the juxtaglomerular apparatus and hilar region into the glomerulus (Hugo et al. 1997). Reminiscent to mesangial cell recruitment during embryonic glomerulogenesis, the involvement of extraglomerular mesangial progenitor cells in glomerular repair was reported by several investigators (Takahashi et al. 1998). The involvement of bone marrow–derived cells in normal mesangial cell turnover was also demoenstrated (Imasawa et al. 2001). Lethally irradiated mice given transplants of T-cell-depleted bone marrow cells from syngeneic donor transgenic for green fluorescent protein (GFP) manifested a time-dependent increase in GFP-positive cells in their glomeruli. When isolated and cultured, these cells stained positive for the mesangial cell marker desmin and the cells contracted in response to angiotensin II, confirming that bone marrow–derived cells have the potential to differentiate into glomerular mesangial cells (Rookmaaker et al. 2004). Similar experiments with mice transplanted with purified clonally expanded hematopoietic progenitor cells were carried out to confirm the hematopoietic origin of bone marrow–derived mesangial cells (Masuya et al. 2003). In similar experiments, using a rat allogenic bone marrow transplant model and antibodies to the mesangial cell–specific antigen Thy-1 (ox7), this time-dependent increase of bone marrow–derived mesangial cells in the glomerulus was confirmed (Rookmaaker et al. 2003). Also, a major increase of bone marrow– derived mesangial cells during recovery from anti-Thy- 1.1–induced mesangiolysis in bone marrow transplantation models in rats was observed (Ito et al. 2001). Cornacchia et al. (2001) demonstrated that glomerulosclerosis can be transmitted by bone marrow transplantation in mice. Transplantation of bone marrow cells from sclerosis-prone mice in normal background mice invoked glomerulosclerosis in the recipients. These data not only point to the contribution of bone marrow–derived cells to glomerular maintenance and repair but also show that dysfunctional or diseased mesangial progenitor cells can have a negative influence on the kidney (Hishikawa and Fujita 2006).

38.3.3 Regeneration of the Renal Tubules

The renal tubule is known for its high capacity for regeneration. Acute tubular necrosis, as a result of ischemia or toxic substances, can be followed by active migration and proliferation to restore normal tissue architecture and function (Toback 1992). Different sources of these proliferating progenitor cells have been

reported. Isolated resident proliferative epithelial cells from the tubuli of mature rabbit kindeys displayed a high capacity for self renewal and differentiated into complete three-dimensional tubular structures in vitro (Humes et al. 1996). Similar experiments were later performed with human epithelial cells (Humes et al. 2004). Bone marrow-derived extrarenal tubular progenitor cells were reported by Poulsom et al.: In the tubules of renal biopsy specimens from eight male patients transplanted with female kidneys, they found Y-chromosome–positive tubular cells within the tubules that co-expressed epithelial markers (Poulsom et al. 2001). However, the proportion of Y-chromosome–positive tubular cells ranged from 1.8 to 20%. Gupta et al. reported finding Ychromosome–positive tubular cells in renal biopsies taken from two men transplanted with female kidneys, but the positive cells made up less than 1% of the tubular cells examined (Gupta et al. 2002). In female mouse recipients of male bone marrow grafts co-localization was observed of Y-chromosomes and tubular epithelial cell markers, suggesting participation of bone marrow-derived cells in normal tubular cell turnover. The potential importance of the role of bone marrow-derived cells in tubular repair was demonstrated by Kale et al. (2003) When LacZ gene-positive bone marrow cells from Rosa26 mice were transplanted into wild-type mice, renal ischemia was associated with the occurence of LacZ-positive (i.e. bone marrow-derived) tubular cells. It was estimated that the majority of the tubular cells after tubular repair were bone marrow–derived. Moreover, bone marrow ablation diminished functional recovery after tubular ischemia, while infusion of a progenitor cell reversed this effect, suggesting an important functional role for the hematopoietic stem cell in tubular repair (Rookmaaker et al. 2004). When male kidney transplant patients who received a female kidney and who recovered from acute tubular necrosis were studied, a Y chromosome could be demonstrated in few (less than 1%) of the tubular cells. Although the functional importance of this phenomenon in the human situation is still uncertain, these experiments do provide us with a proof-of-principle observation on bone marrowderived tubular repair.

38.4 Clinical Implications

Most therapies in nephrology focus on reducing renal damage. However, insight in renal repair and maintenance may offer new therapeutic strategies. Progenitor cells appear to participate in renal repair and turnover of the major renal cell types. Therefore, renal progenitor cells may encompass a new target for therapeutic strategies aimed at the reduction or even prevention of renal disease. Such strategies could be directed toward different populations of progenitor cells. The advantage of circulating progenitor cells may be that they are more accessible for isolation in comparison to the resident progenitor cells. Autologous progenitor cells from the patient are preferable to allogenic progenitor cells because of possible rejection. Obviously, in case of inherited progenitor cell disease, allogenic cells should be considered. One approach to harness progenitor cells for therapeutic purposes is to increase the available pool of progenitor cells. Such expansion may be achieved by growth factor therapy both *in vivo* and *ex vivo*. VEGF and erythropoietin are

probably good candidates to stimulate progenitor cell–mediated endothelial repair. Both have EPC mobilizing and proangiogenetic activities (Asahara et al. 1999; Bahlmann et al. 2003; Heeschen et al. 2003). Another approach to enhance cellular repair and maintenance is reinforcement of progenitor cell function. EPC dysfunction has been shown in diabetic patients (Sorrentino et al. 2007). The decreased re-endothelialization capacity of EPCs from diabetic patients was restored after oral therapy with the PPAR-γ agonist rosiglitazone (Sorrentino et al. 2007).

Mesangial and mesangial progenitor cell dysfunction has been described, too (He et al. 1996; Cornacchia et al. 2001). Replacement of these prosclerotic cells by healthy allogenic mesangial progenitor cells may potentially reduce or even prevent progressive renal disease. The relatively low turnover rate of mesangial cells of 1% per day might however hamper this strategy (Pabst and Sterzel 1983). Controlled mesangial injury by pharmacologic agents combined with healthy allogenic or transfected autologous mesangial precursor cell infusion might increase mesangial turnover and improve cell replacement. Finally, progenitor cells can be used as so-called "magic bullets." (Rookmaaker et al. 2004) Progenitor cells are able to home and participate in their target tissue. This ability can be used to deliver certain gene products very locally. Gene therapy has already successfully been used. Transfection of skeletal muscles with the gene of a transforming growth factor-b1 (TGFb1) inhibitor was able to reduce glomerulosclerosis in a rat nephritis model (Isaka et al. 1996). Transfection of renal progenitor cells might provide a more local therapy, preventing possible systemic side effects.

38.5 Clinical Studies, Experience, Outcome/Side Effects of Kidney Regenerative Therapies

Preclinical studies suggest that the administration of exogenous stem cells may ameliorate acute kidney injury and accelerate regeneration (Table 38.1). In consideration of the role of endogenous bone marrow-derived stem cells, a possible approach could also be stem cell mobilization. However, the possible effects of bone-marrow-recruited cells and of inflammatory cells in this experimental setting require further investigation. Currently, the most promising approach may be the administration of *in vitro* expanded mesenchymal stem cells applied to acute tubular and glomerular injury (Bussolati et al. 2009). Injected mesenchymal stem cells were shown to home to the injured kidney and to accelerate morphological and functional regeneration, possibly by a paracrine or even endocrine mechanisms, although their engraftment and transdifferentiation was not observed in the majority of the studies. A major role in the effect of mesenchymal stem cells has been attributed to the production of growth factors and cytokines with immunosuppressive, antiinflammatory, anti-apoptotic and proliferative effects. Several clinical trials have been designed or are in progress to evaluate the effect of mesenchymal stem cells administration in renal transplantation, acute renal injury or chronic allograph nephropathy (Table 38.2, www.clinicaltrial.gov). The effect of mesenchymal stem cells administration in chronic renal damage still deserves investigation.

Table 38.1 Therapeutic administration of stem cells in experimental animal models of renal damage

Reference	Model	Stem cells	Outcome
Morigi et al. (2004)	Cisplatin ARF	MSC	Improved urea; decreased tubular damage
Togel et al. (2005)	I/R ARF	MSC	Improved creatinine; lower renal injury score
Lange et al. (2005)	I/R ARF	MSC	Improved creatinine; lower renal injury score
Duffield et al. (2005)	I/R ARF	MSC	Improved creatinine
Broekema et al. (2005)	I/R ARF	MSC	Morphological/functional recovery
Bi et al. (2007)	I/R ARF	MSC	Morphological/functional recovery
Kale et al. (2003)	I/R ARF	MSC	Morphological/functional improvement
Herrera et al. (2004, 2007a)	Glycerol ARF	MSC	Improved creatinine; decreased tubular damage
Ninichuk et al. (2006)	$^{5}/_{6}$ nephrectomy	MSC	Decreased interstitial fibrosis
Kunter et al. (2006)	MPGN (Anti-Thy1.1)	MSC	Decreased mesangiolysis, improved creatinine and decreased proteinuria
Uchimura et al.(2005)	MPGN (Anti-Thy1.1)	MSC	Decreased glomerular injury score
Wong et al. (2008)	MPGN (Anti-Thy1.1)	MSC	Decreased glomerular injury score
Rookmaaker et al. (2002, 2007)	MPGN (Anti-Thy1.1)	MSC, EPC	Recovery and vascularization
Prodromidi et al. (2006)	Alport	MSC	Improved renal function, decreased glomerular scarring and interstitial fibrosis
Sugimoto et al. (2006)	Alport	MSC	Partial restoration of expression of type IV collagen α3 chain with concomitant α4 and α5 chain expression, improved glomerular architecture, reduction of proteinuria
Chade et al. (2009)	Renal artery stenosis	EPC	Decreased microvascular remodeling, preserved microvascular architecture

ARF acute renal failure, *MSC* mesenchymal stem cells, *I/R* ischemia/reperfusion, *MPGN* mesangioproliferative glomerulonephritis, *EPC* endothelial progenitor cells

Table 38.2 Current clinical trials using stem cells to treat kidney disease

Title	Disease	Cells	Trial	Time	Clinicaltrials.gov identifier
Allogenic multipotent stromal cell treatment for acute kidney injury following cardiac surgery	Acute renal failure	MSC	Phase I, non-randomized, open label, single group assignment	8/2008–7/2009	NCT00733876
Effect of mesenchymal stem cell transplantation for lupus nephritis	Lupus nephritis	MSC	Phase I +II, open label, active control, single group assignment	5/2008–5/2010	NCT00659217
Mesenchymal stem cell transplantation in recipients of living kidney allografts	Kidney Transplantation	MSC	Randomized, open label, active control, parallel assignment	3/2008–3/2009	NCT00658073
Mesenchymal stem cell transplantation in the treatment of chronic allograft nephropathy	Chronic allograft nephropathy	MSC	Phase I +II, open label, historical control, single group assignment	5/2008–5/2010	NCT00659620

38.6 Conclusions and Future Perspectives on Kidney Regenerative Therapies

Acute and chronic kidney diseases have a complex pathophysiology that may involve both ischemic and inflammatory as well as immunological injury. In contrast to most current pharmacological agents that target only a single pathophysiological pathway, cell-based therapies such as mesenchymal stem cells act through multiple mechanisms and have the potential to target immunological, vascular and inflammatory pathways. In addition, mesenchymal stem cells have the capacity to engraft and survive long-term in a specific target tissue and are both non-immunogenic and immunosuppressive. This has important implications for the therapeutic application of mesenchymal stem cells in tissue repair and regeneration, in that mesenchymal stem cells derived from healthy unrelated volunteer donors can be cryopreserved, thus making them available in a timely manner for patients in a variety of acute and chronic clinical settings. The clinical application of mesenchymal stem cells is broad and has generated significant interest in clinicians from diverse fields, with preclinical and clinical data in a wide variety of conditions, including osteogenesis imperfecta, osteoarthritis and cardiac regeneration. Mesenchymal stem cells are currently being used in hematopoietic stem cell transplantation and although numbers to date are small, the results in high-risk populations with severe graft versus host disease are encouraging. However, many questions remain about their basic biology and long-term safety. More research is needed to understand the physiological role of these cells, their stimuli for migration and the pathways that mediate their apparent beneficial effects in regeneration and repair. Protocols that limit the differentiation potential of the cells into a specific lineage when used for treatment of a specific disease are needed, along with studies that determine the correct dose, schedule and administration route. Despite the lack of apparent adverse effects seen in trials to date, longer-term follow up is required given the possibility of malignant transformation (McTaggart and Atkinson 2007).

And although there is excitement about the application of many of these novel regenerative approaches, many hurdles remain. The unique architecture of the kidney creates substantial obstacles to the functional integration of a stem cell–derived nephron. Indeed, the functional capacity of a bioengineered organ to provide anything like the filtering and resorptive capacity of the endogenous kidney is doubtful (Little 2006).

The final major obstacle is the degree of damage that is present in a patient with chronic renal disease. It is unlikely than any organ-based repair process will overcome the extent of damage that is seen in a patient who has reached end-stage renal failure. This has major implications for the adoption of any autologous therapy. Even if an adult stem cell population does exist in the adult kidney, would it remain in an end-stage kidney? Indeed, the adoption of any organ-based cellular therapy is likely to succeed only if chronic renal disease can be diagnosed early and if such therapies are implemented well before end-stage renal failure is reached. As we move closer to that point in time, the ethical debate about whether trials can proceed before end-stage renal disease will become critical. A lack of surrogate end points

with which to assess the success of a cellular therapy in renal disease will make clinical trials long and expensive, eroding the will of the developers to continue to support the trials (Little 2006).

However, the imperative to continue to forge such novel approaches is clear from the rate at which the incidence of chronic renal failure is rising in both the developed and the developing world. In the end, it is unlikely that any such therapies will produce a physiologic outcome that is equivalent to that of a healthy kidney, but as patient numbers inevitably increase the use of dialysis for treatment, a novel therapy that creates an improvement over dialysis will become not only a major achievement but also a necessity.

38.7 Recent Developments

During the last 2 years several areas of renal regeneration have been further developed. A major achievement has been the demonstration of regeneration in the fish. Mammals can partly repair their nephrons, but cannot form new ones. By contrast, fish add nephrons throughout their lifespan and regenerate nephrons *de novo* after injury providing a model for understanding how mammalian renal regeneration may be therapeutically activated. We have shown in the shark kidney that specific stem cells are the main source for regenerating renal tissue. Davidson et al. trace the source of new nephrons in the adult zebrafish to small cellular aggregates containing nephron progenitors. Transplantation of single aggregates comprising 10–30 cells is sufficient to engraft adults and generate multiple nephrons. Serial transplantation experiments to test self-renewal revealed that nephron progenitors are long-lived and possess significant replicative potential, consistent with stem-cell activity. Transplantation of mixed nephron progenitors tagged with either green or red fluorescent proteins yielded some mosaic nephrons, indicating that multiple nephron progenitors contribute to a single nephron. Consistent with this, live imaging of nephron formation in transparent larvae showed that nephrogenic aggregates form by the coalescence of multiple cells and then differentiate into nephrons. Taken together, these data demonstrate that the zebrafish kidney probably contains self-renewing nephron stem/progenitor cells. The identification of these cells paves the way to isolating or engineering the equivalent cells in mammals and developing novel renal regenerative therapies.

Another area is the identification of novel molecules which regulate renal repair and regeneration. Molecules associated with the transforming growth factor β (TGF-β) superfamily, such as bone morphogenic proteins (BMPs) and TGF-β, are key regulators of inflammation, apoptosis and cellular transitions. Kalluri and his colleagues have shown that the BMP receptor activin-like kinase 3 (Alk3) is elevated early in diseased kidneys after injury. They also found that its deletion in the tubular epithelium leads to enhanced TGF-β1-Smad family member 3 (Smad3) signaling, epithelial damage and fibrosis, suggesting a protective role for Alk3-mediated signaling in the kidney. A structure-function analysis of the BMP-Alk3-BMP receptor, type 2

(BMPR2) ligand-receptor complex, along with synthetic organic chemistry, led them to construct a library of small peptide agonists of BMP signaling that function through the Alk3 receptor. One such peptide agonist, THR-123, suppressed inflammation, apoptosis and the epithelial-to-mesenchymal transition program and reversed established fibrosis in five mouse models of acute and chronic renal injury. THR-123 acts specifically through Alk3 signaling, as mice with a targeted deletion for Alk3 in their tubular epithelium did not respond to therapy with THR-123. Combining THR-123 and the angiotensin-converting enzyme inhibitor captopril had an additive therapeutic benefit in controlling renal fibrosis. Their studies show that BMP signaling agonists constitute a new line of therapeutic agents with potential utility in the clinic to induce regeneration, repair and reverse established fibrosis.

A fascinating area of kidney regeneration is the use of progenitor cells fort he repair and regeneration of damaged renal tissue. There is a pressing need for improved strategies to arrest or reverse intra-renal injury in kidneys with chronically impaired blood flow. Endogenous endothelial progenitor cells (EPC) are often mobilized to mediate neovascularization and endothelial replacement that contribute to healing ischemic tissues. The mobilization from bone marrow and subsequent homing of progenitor cells can be regulated by a variety of mediators such as stromal cell-derived factor (SDF)-1, stem cell factor (SCF), erythropoietin (EPO), or angiopoietins, which are released by injured tissue to attract the cells and ensure their adherence. In turn, the cells express corresponding cognate receptors such as CXCR4, cKit, EPO-receptors (EPO-R), and Tie, respectively, which allow them to be recognized, recruited, and retained at the injured tissues.

However, the endogenous system may be overwhelmed or dysfunctional, and hence fail to repair the tissues. Therefore, exogenous delivery of EPC collected and expanded *in-vitro* offers the potential for targeted treatment of conditions such as chronicallydamaged kidneys. Lerman and co-workers have recently shown the beneficial effects of intra-renal administration of autologous EPC in a porcine model of chronic non-atherosclerotic RAS. Conceivably, a decrease in tissue damage may resolve the injury signals and homing cues that it releases.

Specific signals that portend chronic ischemic injury and regulate the homing and adherence of endogenous circulating cells into the ischemic kidney, or the ability of successful renal repair to alleviate these signals, have not been elucidated. Lerman et al. tested in an experimental study the hypotheses that, firstly, renovascular disease activates homing signals detectable in both the ischemic kidney and EPC, and secondly, that these signals are attenuated upon renal repair using selective intra-renal cell-based therapy. For this purpose they utilized a pig model of experimental atherosclerotic RAS (ARAS), which recapitulates many characteristics of early human atherosclerotic renovascular diseases. Pigs were treated with intra-renal autologous EPC after 6 weeks of ARAS. Four weeks later, expression of homing-related signals in EPC and kidney, single-kidney function, microvascular density, and morphology were compared to untreated ARAS and normal control pigs. Compared to normal EPC, EPC from ARAS pigs showed increased stromal cell-derived factor (SDF)-1, angiopoietin-1, Tie-2, and ckit expression, but downregulation of erythropoietin and its receptor. The ARAS kidney released the ckit-ligand

stem-cell factor (SCF), uric acid, and erythropoietin, and upregulated integrin β2, suggesting activation of corresponding homing signaling. However, angiopoietin-1 and SDF-1/CXCR4 were not elevated. Administration of EPC into the stenotic kidney restored angiogenic activity, improved microvascular density, renal hemodynamics and function, decreased fibrosis and oxidative stress, and attenuated endogenous injury signals.

38.8 Conclusion

The ARAS kidney releases specific homing signals corresponding to cognate receptors expressed by EPC. EPC show plasticity for organ-specific recruitment strategies, which are upregulated in early atherosclerosis. EPC are renoprotective as they attenuated renal dysfunction and damage in chronic ARAS, and consequently decreased the injury signals. Importantly, manipulation of homing signals may potentially allow therapeutic opportunities to increase endogenous EPC recruitment.

These studies may allow novelclinical studies wherebyEPC are used in patients after opening an atherosclerotic renalartery to enhance the regeneration of the damaged and destroyedtissue. The identification of intra-renal stem cells, novel molecules which regulate tissue repair and the use of progenitor cells may be the areas where clinically relevant progress for the repair and regeneration of renal tissue will be made.

References

Abouna GM, Al-Adnani MS, Kremer GD, Kumar SA, Daddah SK, Kusma G (1983) Reversal of diabetic nephropathy in human cadaveric kidneys after transplantation into non-diabetic recipients. Lancet 2:1274–1276

Asahara T, Takahashi T, Masuda H et al (1999) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 18:3964–3972

Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA (2002) Myogenic specification of side population cells in skeletal muscle. J Cell Biol 159:123–134

Bahlmann FH, DeGroot K, Duckert T et al (2003) Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin. Kidney Int 64:1648–1652

Barasch J, Pressler L, Connor J, Malik A (1996) A ureteric bud cell line induces nephrogenesis in two steps by two distinct signals. Am J Physiol 271:F50–F61

Bi B, Schmitt R, Israilova M, Nishio H, Cantley LG (2007) Stromal cells protect against acute tubular injury via an endocrine effect. J Am Soc Nephrol 18:2486–2496

Broekema M, Harmsen MC, Koerts JA et al (2005) Determinants of tubular bone marrow-derived cell engraftment after renal ischemia/reperfusion in rats. Kidney Int 68:2572–2581

Bussolati B, Bruno S, Grange C et al (2005) Isolation of renal progenitor cells from adult human kidney. Am J Pathol 166:545–555

Bussolati B, Hauser PV, Carvalhosa R, Camussi G (2009) Contribution of stem cells to kidney repair. Curr Stem Cell Res Ther 4:2–8

Cha JH, Kim YH, Jung JY, Han KH, Madsen KM, Kim J (2001) Cell proliferation in the loop of henle in the developing rat kidney. J Am Soc Nephrol 12:1410–1421

Chade AR, Zhu X, Lavi R et al (2009) Endothelial progenitor cells restore renal function in chronic experimental renovascular disease. Circulation 119:547–557

Chade AR, Zhu XY, Krier JD, Jordan KL, Textor SC, Grande JP, Lerman A, Lerman LO (2010) Endothelial progenitor cells homing and renal repair in experimental renovascular disease. Stem Cells 28(6):1039–1047

Cornacchia F, Fornoni A, Plati AR et al (2001) Glomerulosclerosis is transmitted by bone marrow-derived mesangial cell progenitors. J Clin Invest 108:1649–1656

Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM (1989) Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell 57:201–209

Davies JA, Bard JB (1996) Inductive interactions between the mesenchyme and the ureteric bud. Exp Nephrol 4:77–85

Diep CQ, Ma D, Deo RC, Holm TM, Naylor RW, Arora N, Wingert RA, Bollig F, Djordjevic G, Lichman B, Zhu H, Ikenaga T, Ono F, Englert C, Cowan CA, Hukriede NA, Handin RI, Davidson AJ (2011) Identification of adult nephron progenitors capable of kidney regeneration in zebrafish. Nature 470(7332):95–100

Dressler GR (1997) Genetic control of kidney development. Adv Nephrol Necker Hosp 26:1–17

Dressler GR, Deutsch U, Chowdhury K, Nornes HO, Gruss P (1990) Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. Development 109:787–795

Duffield JS, Bonventre JV (2005) Kidney tubular epithelium is restored without replacement with bone marrow-derived cells during repair after ischemic injury. Kidney Int 68:1956–1961

Easterday MC, Dougherty JD, Jackson RL et al (2003) Neural progenitor genes. Germinal zone expression and analysis of genetic overlap in stem cell populations. Dev Biol 264:309–22

Ebrahimi B, Li Z, Eirin A, Zhu XY, Textor SC, Lerman LO (2012) Addition of endothelial progenitor cells to renal revascularization restores medullary tubular oxygen consumption in swine renal artery stenosis. Am J Physiol Renal Physiol 302:F1478

Ekblom P (1989) Developmentally regulated conversion of mesenchyme to epithelium. FASEB J 3:2141–2150

Elger M, Hentschel H, Litteral J, Wellner M, Kirsch T, Luft FC, Haller H (2003) Nephrogenesis is induced by partial nephrectomy in the elasmobranch Leucoraja erinacea. J Am Soc Nephrol 14(6):1506–1518

El-Nahas AM (2003) Plasticity of kidney cells: role in kidney remodeling and scarring. Kidney Int 64:1553–1563

Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183:1797–1806

Gupta S, Verfaillie C, Chmielewski D, Kim Y, Rosenberg ME (2002) A role for extrarenal cells in the regeneration following acute renal failure. Kidney Int 62:1285–1290

Hatini V, Huh SO, Herzlinger D, Soares VC, Lai E (1996) Essential role of stromal mesenchyme in kidney morphogenesis revealed by targeted disruption of Winged Helix transcription factor BF-2. Genes Dev 10:1467–1478

He C, Esposito C, Phillips C et al (1996) Dissociation of glomerular hypertrophy, cell proliferation, and glomerulosclerosis in mouse strains heterozygous for a mutation (Os) which induces a 50% reduction in nephron number. J Clin Invest 97:1242–1249

Heeschen C, Aicher A, Lehmann R et al (2003) Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood 102:1340–1346

Herrera MB, Bussolati B, Bruno S, Fonsato V, Romanazzi GM, Camussi G (2004) Mesenchymal stem cells contribute to the renal repair of acute tubular epithelial injury. Int J Mol Med 14:1035–1041

Herrera MB, Bussolati B, Bruno S et al (2007) Exogenous mesenchymal stem cells localize to the kidney by means of CD44 following acute tubular injury. Kidney Int 72:430–441

Herzlinger D (1994) Renal stem cells and the lineage of the nephron. Annu Rev Physiol 56:671–689

Herzlinger D, Koseki C, Mikawa T, al-Awqati Q (1992) Metanephric mesenchyme contains multipotent stem cells whose fate is restricted after induction. Development 114:565–572

Hishikawa K, Fujita T (2006) Stem cells and kidney disease. Hypertens Res 29:745–749
Hishikawa K, Marumo T, Miura S et al (2005a) Leukemia inhibitory factor induces multi-lineage differentiation of adult stem-like cells in kidney via kidney-specific cadherin 16. Biochem Biophys Res Commun 328:288–291
Hishikawa K, Marumo T, Miura S et al (2005b) Musculin/MyoR is expressed in kidney side population cells and can regulate their function. J Cell Biol 169:921–928
Horster M (1985) Ontogenetic processes in nephron epithelia: function, enzymes, and structure. In: Giebisch DWSG (ed) The kidney: physiology and pathophysiology. Raven, New York
Horster MF, Braun GS, Huber SM (1999) Embryonic renal epithelia: induction, nephrogenesis, and cell differentiation. Physiol Rev 79:1157–1191
Hugo C, Shankland SJ, Bowen-Pope DF, Couser WG, Johnson RJ (1997) Extraglomerular origin of the mesangial cell after injury. A new role of the juxtaglomerular apparatus. J Clin Invest 100:786–794
Humes HD, Krauss JC, Cieslinski DA, Funke AJ (1996) Tubulogenesis from isolated single cells of adult mammalian kidney: clonal analysis with a recombinant retrovirus. Am J Physiol 271:F42–F49
Humes HD, Weitzel WF, Bartlett RH et al (2004) Initial clinical results of the bioartificial kidney containing human cells in ICU patients with acute renal failure. Kidney Int 66:1578–1588
Imasawa T, Utsunomiya Y, Kawamura T et al (2001) The potential of bone marrow-derived cells to differentiate to glomerular mesangial cells. J Am Soc Nephrol 12:1401–1409
Iruela-Arispe L, Gordon K, Hugo C et al (1995) Participation of glomerular endothelial cells in the capillary repair of glomerulonephritis. Am J Pathol 147:1715–1727
Isaka Y, Brees DK, Ikegaya K et al (1996) Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney. Nat Med 2:418–423
Ito T, Suzuki A, Imai E, Okabe M, Hori M (2001) Bone marrow is a reservoir of repopulating mesangial cells during glomerular remodeling. J Am Soc Nephrol 12:2625–2635
Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, Lemischka IR (2002) A stem cell molecular signature. Science 298:601–604
Jackson KA, Majka SM, Wang H et al (2001) Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 107:1395–1402
Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisen J (1999) Identification of a neural stem cell in the adult mammalian central nervous system. Cell 96:25–34
Kale S, Karihaloo A, Clark PR, Kashgarian M, Krause DS, Cantley LG (2003) Bone marrow stem cells contribute to repair of the ischemically injured renal tubule. J Clin Invest 112:42–49
Kitamura S, Yamasaki Y, Kinomura M et al (2005) Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney. FASEB J 19:1789–1797
Koseki C, Herzlinger D, al-Awqati Q (1991) Integration of embryonic nephrogenic cells carrying a reporter gene into functioning nephrons. Am J Physiol 261:C550-4
Koseki C, Herzlinger D, al-Awqati Q (1992) Apoptosis in metanephric development. J Cell Biol 119:1327–1333
Kunter U, Rong S, Djuric Z et al (2006) Transplanted mesenchymal stem cells accelerate glomerular healing in experimental glomerulonephritis. J Am Soc Nephrol 17:2202–2212
Lagaaij EL, Cramer-Knijnenburg GF, van Kemenade FJ, van Es LA, Bruijn JA, van Krieken JH (2001) Endothelial cell chimerism after renal transplantation and vascular rejection. Lancet 357:33–37
Lange C, Togel F, Ittrich H et al (2005) Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. Kidney Int 68:1613–1617
Lavker RM, Sun TT (2000) Epidermal stem cells: properties, markers, and location. Proc Natl Acad Sci U S A 97:13473–13475
Little MH (2006) Regrow or repair: potential regenerative therapies for the kidney. J Am Soc Nephrol 17:2390–2401
Majka SM, Jackson KA, Kienstra KA, Majesky MW, Goodell MA, Hirschi KK (2003) Distinct progenitor populations in skeletal muscle are bone marrow derived and exhibit different cell fates during vascular regeneration. J Clin Invest 111:71–79
Masuya M, Drake CJ, Fleming PA et al (2003) Hematopoietic origin of glomerular mesangial cells. Blood 101:2215–2218

McTaggart SJ, Atkinson K (2007) Mesenchymal stem cells: immunobiology and therapeutic potential in kidney disease. Nephrology (Carlton) 12:44–52
Morigi M, Imberti B, Zoja C et al (2004) Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. J Am Soc Nephrol 15:1794–1804
Ninichuk V, Gross O, Segerer S et al (2006) Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. Kidney Int 70:121–129
Oliver JA, Maarouf O, Cheema FH, Martens TP, Al-Awqati Q (2004) The renal papilla is a niche for adult kidney stem cells. J Clin Invest 114:795–804
Olmsted-Davis EA, Gugala Z, Camargo F et al (2003) Primitive adult hematopoietic stem cells can function as osteoblast precursors. Proc Natl Acad Sci U S A 100:15877–15882
Pabst R, Sterzel RB (1983) Cell renewal of glomerular cell types in normal rats. An autoradiographic analysis. Kidney Int 24:626–631
Poulsom R, Forbes SJ, Hodivala-Dilke K et al (2001) Bone marrow contributes to renal parenchymal turnover and regeneration. J Pathol 195:229–235
Prodromidi EI, Poulsom R, Jeffery R et al (2006) Bone marrow-derived cells contribute to podocyte regeneration and amelioration of renal disease in a mouse model of Alport syndrome. Stem Cells 24:2448–2455
Qiao J, Cohen D, Herzlinger D (1995) The metanephric blastema differentiates into collecting system and nephron epithelia in vitro. Development 121:3207–3214
Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA (2002) "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 298:597–600
Risau W (1997) Mechanisms of angiogenesis. Nature 386:671–674
Rookmaaker MB, Tolboom H, Goldschmeding R, Zwaginga JJ, Rabelink TJ, Verhaar MC (2002) Bone-marrow-derived cells contribute to endothelial repair after thrombotic microangiopathy. Blood 99:1095
Rookmaaker MB, Smits AM, Tolboom H et al (2003) Bone-marrow-derived cells contribute to glomerular endothelial repair in experimental glomerulonephritis. Am J Pathol 163:553–562
Rookmaaker MB, Verhaar MC, van Zonneveld AJ, Rabelink TJ (2004) Progenitor cells in the kidney: biology and therapeutic perspectives. Kidney Int 66:518–522
Rookmaaker MB, Verhaar MC, de Boer HC et al (2007) Met-RANTES reduces endothelial progenitor cell homing to activated (glomerular) endothelium in vitro and in vivo. Am J Physiol Renal Physiol 293:F624–F630
Sariola H, Ekblom P, Lehtonen E, Saxen L (1983) Differentiation and vascularization of the metanephric kidney grafted on the chorioallantoic membrane. Dev Biol 96:427–435
Saxen L, Sariola H (1987) Early organogenesis of the kidney. Pediatr Nephrol 1:385–392
Saxen L, Salonen J, Ekblom P, Nordling S (1983) DNA synthesis and cell generation cycle during determination and differentiation of the metanephric mesenchyme. Dev Biol 98:130–138
Sorrentino SA, Bahlmann FH, Besler C et al (2007) Oxidant stress impairs in vivo reendothelialization capacity of endothelial progenitor cells from patients with type 2 diabetes mellitus: restoration by the peroxisome proliferator-activated receptor-gamma agonist rosiglitazone. Circulation 116:163–173
Sugimoto H, Mundel TM, Sund M, Xie L, Cosgrove D, Kalluri R (2006) Bone-marrow-derived stem cells repair basement membrane collagen defects and reverse genetic kidney disease. Proc Natl Acad Sci U S A 103:7321–7326
Sugimoto H, LeBleu VS, Bosukonda D, Keck P, Taduri G, Bechtel W, Okada H, Carlson W Jr, Bey P, Rusckowski M, Tampe B, Tampe D, Kanasaki K, Zeisberg M, Kalluri R (2012) Activin-like kinase 3 is important for kidney regeneration and reversal of fibrosis. Nat Med 18(3):396–404
Takahashi T, Huynh-Do U, Daniel TO (1998) Renal microvascular assembly and repair: power and promise of molecular definition. Kidney Int 53:826–835
Toback FG (1992) Regeneration after acute tubular necrosis. Kidney Int 41:226–246
Togel F, Hu Z, Weiss K, Isaac J, Lange C, Westenfelder C (2005) Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. Am J Physiol Renal Physiol 289:F31–F42

Uchimura H, Marumo T, Takase O et al (2005) Intrarenal injection of bone marrow-derived angiogenic cells reduces endothelial injury and mesangial cell activation in experimental glomerulonephritis. J Am Soc Nephrol 16:997–1004

Williams GM, Alvarez CA (1969) Host repopulation of the endothelium in allografts of kidneys and aorta. Surg Forum 20:293–294

Wong CY, Cheong SK, Mok PL, Leong CF (2008) Differentiation of human mesenchymal stem cells into mesangial cells in post-glomerular injury murine model. Pathology 40:52–57

Zhou S, Schuetz JD, Bunting KD et al (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 7:1028–1034

Chapter 39
Gastrointestinal Tract and Endocrine System

Nonsikelelo Mpofu-Mätzig, Michelle Klose, Elmar Jäckel, Michael P. Manns, and Oliver Bachmann

Abstract The absorption of nutrients in the small intestine and the control of blood glucose levels are crucial to ensure energy homeostasis of the organism. These functions can be severely impaired in diseases like type I diabetes, short bowel syndrome, and inflammatory bowel disease, for which the standard treatment involves either life-long hormone or nutrient replacement, immunosuppression, or organ transplantation, and is often not satisfactory. After outlining the etiology and pathology of these diseases and established as well as experimental approaches, this chapter summarizes recent preclinical and clinical studies on novel therapeutic options relating to regenerative medicine, including growth factors and stem cells.

39.1 Introduction

39.1.1 Type I Diabetes Mellitus

Human type 1 diabetes (T1D) is an autoimmune disease that arises in genetically predisposed individuals due to the destruction of insulin producing beta cells of the pancreatic islet of Langerhans, a process triggered by autoaggressive CD4+ and CD8+ T cells. This results in a lack of control of blood glucose levels culminating in hyperglycemia if more than 90% of beta cells are destroyed. This in time leads to

N. Mpofu-Mätzig, Ph.D. • M. Klose, M.D. • E. Jäckel, M.D.
M.P. Manns, M.D. (✉) • O. Bachmann, M.D.
Department of Gastroenterology, Hepatology and Endocrinology,
Centre for Internal Medicine, Hannover Medical School (MHH),
Carl-Neuberg-Str. 1, 30625 Hannover, Germany
e-mail: manns.michael@mh-hannover.de

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_39,

Table 39.1 Comparison of T1D in NOD mice and human patients

Characteristic	Humans	Mice
Genetic predisposition and polygenetic trait	Yes	Yes
MHC-loci contribution	Multiple	Multiple
Environmental influence	Yes	Yes
Defective peripheral immune regulation	Yes	Yes
Impaired dendritic-cell maturation	Possibly	Possibly
Autoantigens	GAD65, IA2, insulin and Hsp60	GAD65, IA2, insulin and Hsp60
Initiating auto antigen	Unknown	Unknown
Islet auto immunity linked to early gluten exposure	Yes	Yes

Adapted from Roep et al. (2004)

severe chronic complications such as widespread microvascular (retinopathy, nephropathy and neuropathy) and macrovascular damage (myocardial infarction, stroke, peripheral arterial vascular disease) (Atkinson and Maclaren 1994; Daneman 2006). The search for a cure for T1D or effective treatments that can prevent the complications associated with T1D is still ongoing.

T1D accounts for 5–10% of all diabetes cases and commonly occurs in people of European descent with estimates of 2 million people affected in Europe and North America (Daneman 2006; Gillespie 2006). T1D is largely known as a childhood disease accounting for 90% of childhood-onset diabetes (Daneman 2006; von Herrath et al. 2007). It has been reported that the incidence of T1D is on the rise with predictions suggesting a doubling of new cases in European children under the age of 5 years by the year 2020, with the prevalence of cases expected to rise by 70% in individuals that are below 15 years of age (Gillespie 2006; Patterson et al. 2009).

39.1.2 Etiology of T1D

Much of our understanding of the etiology and pathogenesis of T1D stems from research conducted in the spontaneous non obese diabetic (NOD) mouse and biobreeding rat (BB rat) rodent models of T1D. The NOD mouse is the most widely used animal model of T1D that shares major disease characteristics with human disease as shown in Table 39.1 (Atkinson and Leiter 1999; Anderson and Bluestone 2005).

Studies conducted in the NOD mouse show that like in humans, T1D is a complex disease that is precipitated by a combination of factors such as genetic susceptibility, environmental triggers and immune dysregulation (Kishimoto and Sprent 2001; Anderson and Bluestone 2005; Daneman 2006).

Genetic susceptibility to T1D is heritable and lies predominantly within the major histocompatibility (MHC) or human leukocyte antigen (HLA) locus in the NOD mouse and human disease respectively (Anderson and Bluestone 2005). MHC/HLA molecules function in the initiation of immune responses to foreign antigens by presenting antigens to T cells bearing the respective T cell receptor (TCR) specificity. Furthermore these molecules are also involved in tolerance induction to self-antigens and account for both positive and negative selection of autoreactive T cells within the thymus. Therefore the occurrence of allelic variability within the MHC/HLA loci may lead to immune dysregulation. In human disease, strong associations have been made between T1D susceptibility and genes located within the HLA-DR and HLA-DQ loci (Cucca et al. 1993; Noble et al. 1996). In addition, the risk assessment of familial T1D can be conducted by screening for allelic variations within the HLA-DR and HLA-DQ loci (Nejentsev et al. 1999; Ilonen et al. 2002).

Variability within the insulin gene, in particular a variable number tandem repeat in the insulin promoter (insulin-VNTR), has been shown to also contribute to disease susceptibility, albeit to a lesser extent than the MHC/HLA. The insulin-VNTR controls the expression of insulin in the thymus, therefore potentially regulating the autoimmune repertoire. Shorter forms of insulin–VNTR are reported to be associated with T1D development, whilst the longer forms are associated with greater protection of thymic insulin message correlating with T1D protection (Bluestone et al. 2010; Gillespie 2006).

Disease susceptibility has been further associated with the global immune dysregulation that occurs in both the NOD mouse and human disease. Indeed genetic variations in genes associated with immune homeostasis such as the regulatory cytotoxic T-lymphocyte antigen 4 (CTLA-4), the co-stimulatory molecule CD28, PTPN22 (which encodes the lymphoid protein tryrosine phosphate – LYP), IL-2RA (CD25), and PD-1 have been demonstrated to be associated with T1D development. Proteins encoded by these genes maintain homeostasis by either activating T and B cells or regulating their activity through regulatory cell populations which is key to regulating autoimmunity (Bluestone et al. 2010; van Belle et al. 2011). On the whole, genetic susceptibility to T1D results from genetic variability in genes that are associated with antigen presentation, central tolerance induction and immune regulation.

Environmental factors are also believed to be involved in the causation of T1D. Indeed, the increase in disease incidence amongst young children has been reported to be occurring rather rapidly to result from genetic alterations alone, thereby implying that environmental factors may be responsible (Gillespie 2006). The question of the identity of the environmental triggers involved is still under debate, though it is widely viewed that viruses such as enteroviruses (particularly Coxsackie B viruses), rubella, and rotaviruses act as environmental triggers for T1D (Ginsberg-Fellner et al. 1985; Peltola et al. 2000). Furthermore, environmental toxins and foods such as cow milk proteins, cereals, or gluten have also been reported to be associated with the causation of T1D (Daneman 2006).

39.1.2.1 Immunopathogenesis of T1D

T1D results from a series of complex events and is believed to be initiated in genetically susceptible individuals by environmental triggers. How environmental factors trigger T1D is still under elucidation. However studies suggest that factors such as virus infection or environmental toxins prompt the up regulation of IFN-γ and MHC class I molecules by pancreatic beta cells. This in turn leads to a loss of tolerance culminating in the release of beta cell antigens such as insulin, glutamic acid decarboxylase 65 (GAD65), the zinc transporter (ZnT8), protein tyrosine phosphatase (IA-2), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), and heat shock protein 60 (Hsp60) (Jaeckel et al. 2008). These beta cell antigens are then taken up by antigen presenting cells and transported to the pancreatic lymph nodes where they are presented to potentially autoreactive T cells.

Studies in the NOD mouse show that dendritic cells and macrophages infiltrate the pancreas in the initial phase of T1D. Shortly thereafter, B cells and potentially autoreactive CD4+ and CD8+ T cells migrate from the pancreatic lymph node and infiltrate the pancreas without initially destroying the beta cells. This stage of the disease is known as insulitis (Gianani and Eisenbarth 2005). At this stage, B cells initiate a humoral response that leads to the production of beta cell autoantibodies. The role of beta cell specific autoantibodies in the pathophysiology of T1D is still controversial, yet they serve as a very useful biomarker for the development of autoimmunity (Waldron-Lynch and Herold 2011; Daneman 2006). Furthermore, insulin-specific autoantibodies can be detected months to years prior to the onset of clinical symptoms. This lag period between initiation of autoimmunity and development of overt diabetes also explains the difficulties in identifying a causative environmental agent for T1D.

After a gradual increase in cellular infiltrates, the progressive destruction of beta cells ensues. At this stage, beta cells are destroyed by perforin, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α), that are produced by the activated CD4+ and CD8+ autoreactive T cells. This leads to the release of new beta-cell antigens that are then taken up by antigen presenting cells including migrated B cells and presented in the pancreatic lymph nodes to new T and B cell specificities. As a result of epitope spreading, new antibody specificities can also be detected, such as antibodies against GAD65, ZnT8 and IA-2. The number of detectable autoantibodies has been used in T1D risk assessment, with patients positive for three or more specificities exhibiting a higher risk of developing diabetes in comparison to patients positive for a single specificity (Waldron-Lynch and Herold 2011; Daneman 2006). Subsequently, the beta cells undergo a more aggressive immune attack that leads to a severe depletion of the beta cell mass. This leads to a complete loss of insulin production and dysregulation of glucose metabolism, a stage known as overt diabetes (van Belle et al. 2011; Gianani and Eisenbarth 2005). Hyperglycemia results as a consequence of insulin deficiency, and T1D is diagnosed when there is about 10–30% of functional beta cells remaining (Gianani and Eisenbarth 2005).

39.1.2.2 Current Therapeutic Options for T1D

Insulin Replacement Therapy

Treatment for T1D currently involves insulin replacement therapy by subcutaneous injections of exogenous insulin. This treatment also requires daily blood glucose level measurements in order to determine the correct dosage of insulin required to control hyperglycemia, and to prevent hypoglycemia. For the best outcome, a multidisciplinary health team is required that also assists with dietary planning and screening for diabetes related complications (Daneman 2006). Although insulin replacement therapy can control hyperglycemia, it does not induce immune tolerance and patients are still in danger of developing microvascular and macrovascular complications of diabetes due to suboptimal glucose control. Furthermore, there is the issue of compliance as patients have to treat themselves for decades beginning from childhood. Side effects such as hypoglycemia, weight gain, and excessive diurnal glucose fluctuations have also been associated with insulin replacement therapy (Waldron-Lynch and Herold 2009). Therefore there is a need for new and potentially curative therapies for T1D that can improve the quality of life for patients.

Pancreas Transplantation

As previously stated, hypoglycemia is a dangerous side effect associated with insulin replacement therapy. This is particularly problematic in a subgroup of patients with erratic glycemic control (i.e. labile diabetes). For these patients whole organ pancreas or allogeneic islet transplantation are an alternative therapeutic approach (Vardanyan et al. 2010).

Since the first pancreas transplant in 1966, more than 30,000 pancreas transplants have been performed worldwide. About 7% of pancreas transplants were single organ transplants, with 72% performed in patients undergoing simultaneous pancreas-kidney(SPK)-, 17% undergoing pancreas after kidney (PAK)- and less than 4% undergoing combined heart, liver or intestine procedures (Gruessner and Sutherland 2008). One year survival rates after transplantation were over 95% and 83% after 5 years. Furthermore, SPK exhibited the best graft survival in comparison to the other procedures (Gruessner and Sutherland 2008). Despite its success, pancreas transplantation is limited by the scarcity of tissue pancreas donors and the side effects of immunosuppressive drugs taken to prevent allograft rejection. Due to the invasive nature of pancreas transplantation, it is limited to a subgroup of individuals with labile diabetes and those undergoing kidney transplantation at the same time. SPK improves survival compared to patients with T1D on dialysis, and in addition, SPK also improves survival of the kidney graft. Despite these facts, just 150–160 patients with T1D received a pancreas transplantation, although an estimated 3,000–5,000 patients with T1D are on hemodialysis.

Islet Cell Transplantation

Due to the limitations of whole organ transplantation, islet transplantation has been considered as an alternative approach to restore normoglycemia. Islet transplantation via percutenous transhepatic portal embolism combined with a corticoid-free immunosuppressive regimen (Edmonton protocol) was demonstrated to result in a 1-year insulin independence in 50–80% of treated patients (Shapiro et al. 2000). Patients exhibited better glycemic control and no hypoglycemic events. However, by year five 90% of patients were again dependent on exogenous insulin, although remaining C-peptide production of the graft could still be observed in many of those patients. The latter result was attributed to the side effects of the immunosuppressive regimen that involved administration of daclizumab (humanized anti-CD25 mAb), rapamycin (sirolimus), and FK-506 (tacrolimus). This regimen was reported to result in lymphopenia and led to the induction of homeostatic cytokines that expanded autoreactive T cells, thereby accounting for recurrent autoimmunity in transplanted patients. Furthermore, rapamycin was shown to impair engraftment, induce insulin resistance and inhibit beta cell replication (Zhang et al. 2006, 2007; Fraenkel et al. 2008; Monti et al. 2008). Clinical trials for islet transplantation using different combinations of immunosuppressive drugs are currently underway (van Belle et al. 2011).

Although promising, islet transplantation is limited by the isolation of adequate islets from the little available donor tissue. In order to meet demand xenogeneic islets especially from pigs have been considered as an alternative source of islets for transplantation. Porcine islets are ideal as they are physiologically similar to human islets and are readily available due to rapid breeding of pigs. However, this procedure is not without its drawbacks such as the possibility of zoonotic infections and an aggressive xenogeneic immune response against the islet xenografts. The latter, however, could potentially be overcome by co-stimulation blockade or encapsulation of the islet xenografts (Cardona et al. 2007; Kobayashi et al. 2008). Initial xenogeneic islet transplantations performed in New Zealand and Moscow, however, proved to be safe as no transmission of porcine pathogens or viruses was observed. The effectivity of the transplant procedure still needs to be determined (Garkavenko et al. 2008).

39.1.2.3 Therapeutic Advances for T1D

Our increased knowledge of the etiology and pathogenesis of T1D has led to the identification of a number of potential targets for therapy. Preclinical and clinical observations strongly suggest that a successful therapeutic strategy for T1D should fulfill three requirements: Firstly, the therapy should be short term and able to re-establish immune tolerance by regulating the ongoing beta cell destruction. Secondly, the therapy must be able to maintain immune tolerance in order to facilitate beta-cell regeneration. And finally, the therapy should be acceptable to T1D patients and should have a similar or even better effect than the existing insulin replacement

therapy (Waldron-Lynch and Herold 2009). In the section below we explore some of the therapeutic advances made thus far and their clinical applications.

Immunomodulatory Therapies

The goal of immunomodulatory therapies is to inhibit the destructive autoimmune response against the pancreatic beta cells in order to preserve and potentially restore beta cell function. Immunomodulatory therapies can be classified as either polyspecific (i.e. use of global or cell targeted immunosuppressants) or antigen specific (mediated by antigen specific tolerance induction).

Polyspecific Immunomodulatory Strategies: Immunosuppressive Drugs

The calcineurin inhibitor cyclosporine A and the corticosteroid prednisone were shown to deplete or inactivate T cells in solid organ transplantation. This led to their use in the initial clinical trials for T1D. Treatment of T1D patients with either cyclosporine A or prednisone in combination with the purine analogue azathioprin induced disease remission, with 50% of patients requiring no exogenous insulin during treatment (Feutren et al. 1986; Stiller et al. 1987; Silverstein et al. 1988). However, treatment with immunosuppressive drugs did not restore tolerance to pancreatic beta cells as disease remission was largely limited to the duration of drug administration. Therefore, maintenance of normoglycemia would mean chronic treatment with these drugs. However, side effects associated with prolonged usage of cyclosporine A and prednisone, such as systemic immunosuppression, induction of nephrotoxicity and insulin resistance prevented their further clinical use (Bougneres et al. 1990; Parving et al. 1999).

Polyspecific Immunomodulatory Strategies: Targeted Cell Therapies

It was shown in the mid-1980s that short term treatment with depleting or non depleting isotypes or F(ab′)2 fragments of CD4 antibodies induces long-term tolerance to skin and islet allografts (Gutstein et al. 1986; Carteron et al. 1989). The results obtained using F(ab′)2 fragments and non-depleting isotypes of CD4 antibodies further demonstrated that non-depleting monoclonal antibodies can be used for tolerance induction *in vivo*. Since then, antibodies against CD40L, CD25, CD3 and CLTA4-Ig amongst others have been shown to facilitate tolerance induction in transplantation and autoimmunity (Waldmann and Cobbold 1998).

Anti-CD3ε Therapy (Teplizumab and Otelixizumab)

One of the first monoclonal antibodies (mAb) described to be effective in preventing organ allograft rejection and in treating acute rejections after transplantation was the anti-CD3 antibody (OKT3, a murine immunoglobulin). However, the success of OKT3 in solid organ transplantation was hampered by development of a severe cytokine

release syndrome that resulted from activation of T cells enhanced by cross-linking the murine Fc portions with human Fc receptors (FcR) (Cosimi et al. 1981; Abramowicz et al. 1989). These findings led to the development of CD3 mAbs, which were humanized making them less immunogenic. In addition the Fc binding site was mutated to prevent cross-linking of Fc-receptors (Friend et al. 1999).

The immunosuppressive capacity of anti-CD3 therapy in T1D was initially tested in the NOD mouse using a brief course of low dose non-Fc binding Fab-fragments of anti-CD3 mAb (145-2C11). These studies showed disease reversal in both treated recent onset and overtly diabetic NOD mice (Chatenoud et al. 1994, 1997). The mechanism of disease reversal by anti-CD3 was shown to involve induction of peripheral tolerance via ignorance due to short term internalization of TCR complex after anti-CD3 binding, induction of anergy or Fas mediated apoptosis of activated Th1 cells and induction of TGF-β dependent adaptive CD4+CD25lowFoxp3+ Tregs from peripheral CD4+CD25- T cells (Chatenoud et al. 1982, 1994; Belghith et al. 2003; You et al. 2007). Owing to the success of the preclinical studies, two non Fc binding anti-CD3 antibodies were used for T1D clinical trials, namely humanized OKT3γ1 (Ala Ala, named teplizumab), and chAglyCD3 (aglycoslated FcR non-binding, named otelixizumab). Administration of a single course of teplizumab or otelixizumab in recent onset patients halted disease progression, and these patients exhibited better preservation of stimulated C-peptide levels and lower insulin usage compared to control groups. This effect lasted up to 4 years after treatment. Furthermore, patients that had the highest endogenous insulin production at the commencement of the clinical trial exhibited the greatest effect. However, despite the promising clinical outcome, the effect of teplizumab and otelixizumab was short-lived. This implies either disease re-emergence or a limitation of drug efficacy to time of disease onset. Recently, phase III clinical trials with teplizumab and otelixizumab were declared a failure owning to their inability to meet their primary endpoints, that is reduced insulin usage and serum HbA1c levels. Furthermore, the phase III trial using otelixizumab failed because of 90% dose reduction between phase II and III trials (Herold and Bluestone 2011; Waldron-Lynch and Herold 2011). However, it has to be emphasized that teplizumab could demonstrate its ability to stabilize stimulated C-peptide secretion repeatedly. Taken together both antibodies are still promising partners for combination therapies described below.

CTLA4-Ig (Abatacept and Belatacept)

The activation of T cells requires two signals: The first signal emerges as a consequence of antigen recognition of MHC-peptide complexes by the TCR while the second signal emanates from the recognition of co-stimulatory molecules (e.g. CD28, CD40L, ICOS) expressed on T cells and their receptors (e.g. B7.1/B7.2, CD40, ICOSL) expressed on activated antigen presenting cells. The best characterized costimulatory pathway is the CD28/B7 pathway. Conversely, the binding of the T cell surface molecule CTLA-4 to B7.1/B7.2 results in negative regulation of T cell activation.

CTLA4-Ig is a fusion protein consisting of a CTLA-4 extracellular domain and an IgG Fc domain. CTLA4-Ig has a higher affinity for B7 molecules than for CD28

and hence acts as a competitive inhibitor of the CD28/B7 pathway. CTLA4-Ig (abatacept) in combination with methotrexate has been shown to be immunosuppressive in rheumatoid arthritis patients. However, abatacept was shown to be non-tolerogenic and patients require monthly infusions to maintain immunosuppression (Waldron-Lynch and Herold 2011; Kremer et al. 2008). On the other hand, administration of CTLA4-Ig in preclinical trials for T1D yielded conflicting results. CTLA4-Ig was shown to prevent diabetes development in an adoptive transfer model of diabetes. In these studies, tolerance induction mediated by expansion of Tregs was observed in treated mice (Rigby et al. 2008). However, in an islet transplantation model, anti-CD4 mAbs were shown to be more effective at preventing disease resurgence than CTLA4-Ig. This could be attributed to the fact that some studies have shown that the CD28/B7 pathway is important for Treg development and survival, and hence administration of CTLA4-Ig may interfere with Treg homeostasis (Guo et al. 2001; Salomon and Bluestone 2001). Recently, a phase II trial using abatacept did just initially (3–6 months) slow the loss of β-cells despite continued use for 24 months. Additionally, belatacept (a high affinity variant of CTLA4-Ig) is currently being tested in a phase I/II islet transplantation trial (van Belle et al. 2011). Taken together, CTAL4-Ig does not seem to be a promising partner for combination therapy in new onset T1D.

Anti-CD20 (Rituximab)

The role of B cells in the pathogenesis of T1D has been overshadowed by the predominant role of T cells. However, evidence of detectable autoantibodies prior to disease onset suggests that B cells may play a role in disease initiation. The first studies to demonstrate a role of B cells in disease pathogenesis were conducted in NOD mice engineered to express a humanized form of the B cell surface molecule CD20 (hCD20). These studies showed that depletion of B cells with an anti-hCD20 antibody resulted in a delay in disease onset and also managed to control already established diabetes. The efficacy of this treatment was attributed to the expansion of regulatory T and B cells (Hu et al. 2007).

Successful treatment of autoimmunity with the anti-CD20 drug (rituximab) was demonstrated in clinical trials for rheumatoid arthritis and systemic lupus. Conversely, disease remission was limited to drug administration bringing to question long-term tolerance induction by rituximab in human disease (Kazkaz and Isenberg 2004; Looney 2005). A phase II clinical trial for T1D using rituximab showed some preservation of C-peptide levels and reduced insulin usage between 3–6 months after treatment. However, the effect of rituximab on T1D was short lived and modest (Waldron-Lynch and Herold 2011).

Antigen Specific Strategies

The rationale behind antigen specific immunomodulatory strategies is based on the evidence that oral, intranasal, or subcutaneous administration of antigens can induce peripheral immune tolerance. The therapeutic capacity of major beta cell antigens

such as insulin, GAD65 and Hsp60 was tested in the preclinical NOD mouse with much success (Atkinson et al. 1990; Zhang et al. 1991; Muir et al. 1995; Daniel and Wegmann 1996; Tian et al. 1996b; Bockova et al. 1997; Elias et al. 1997; Ma et al. 1997). Owing to the success of these preclinical studies, antigen specific immunomodulation for T1D was translated to clinical trials. Furthermore, antigen specific therapies were favored as they can ensure a tissue specific response, which would circumvent the problem of systemic immunosuppression observed by the use of global immunosuppressants.

Insulin Trials

Insulin is considered to be a major autoantigen in both the NOD model and in human T1D. Ins B9-23, a peptide that is recognized by both CD4+ and CD8+ T cells, has been shown to be a prerequisite for T1D development in NOD mice and a target of autoreactive CD4+ T cells in T1D patients (Nakayama et al. 2005). Oral and intranasal administration of insulin prevented disease development in the prediabetic NOD mouse through the induction of Th2 (IL-4/IL-10), Th3-(TGF-β) secreting, CD8+ and IL-10 dependent Tr-1 regulatory T cell populations (Atkinson et al. 1990; Zhang et al. 1991; Daniel and Wegmann 1996; Harrison et al. 1996; Faria and Weiner 2006a, b). However, parenteral and intranasal administration of insulin in at risk patients did not have an effect on disease progression in prediabetic and recent onset patients (Pozzilli et al. 2000, Pozzilli 2002; Kupila et al. 2003; Harrison et al. 2004). A beneficial effect was however observed in a subgroup of patients that had high titers of insulin autoantibodies. The results were sustained over 8 years in patients on continued therapy (Vehik et al. 2011).

GAD65 Trials

Glutamic acid decarboxylase (GAD) is an enzyme that exists in two isoforms (GAD65 and GAD67) and is involved in the production of the neurotransmitter γ-aminobutyric acid (GABA). GAD is expressed exclusively in the brain and pancreas. GAD65 reactivity has been primarily associated with T1D with the presence of GAD65 reactive T cells and autoantibodies reported in T1D patients and at risk individuals (Fenalti and Rowley 2008). Intranasal and intravenous administration GAD65 in prediabetic or recent onset NOD mice resulted in disease prevention that was mediated by antigen specific CD4+ regulatory T cells with a Th2 phenotype (Tian et al. 1996a, b; Tisch et al. 1998, 1999; Chen et al. 2003). Administration of Diamyd (recombinant human GAD65 formulated in alum) in patients with latent autoimmune diabetes in adults (LADA) or recent onset T1D resulted in the preservation of insulin secretion. The effect of GAD alum treatment was attributed to the induction of TGF-β secreting FOXP3+ regulatory T cells (Hjorth et al. 2011; Agardh et al. 2005, 2009; Ludvigsson et al. 2008). Unfortunately, two recently conducted Phase III clinical trials in Europe and the USA did not reproduce these effects and failed (Wherrett et al. 2011). However, due to the low side effects of GAD65 administration it might be a potential candidate for combination therapy in GADA positive patients.

DiaPep277 Trials

p277 is a major T cell epitope of the heat shock protein60 (HSp60) and has been shown to be an immunodominant epitope in human and NOD type 1 diabetes (Horvath et al. 2002). Additionally murine and human p277 have been shown to differ in a single position and NOD T cells have been shown to respond to stimulation with the human peptide (Birk et al. 1996; Horvath et al. 2002). Furthermore, subcutaneous vaccination of the human p227 prevented diabetes in recent onset and overtly diabetic NOD mice via a Th2 mediated cytokine burst and induction of Qa-1 CD8+ regulatory T cells (Elias and Cohen 1994; Bockova et al. 1997; Elias et al. 1997).

Administration of DiaPep277 in phase II trials, a modified version of the p277 in which cysteines were substituted with valine in order to improve stability, resulted in the preservation of C-peptide for up to 18 months in adult recent onset diabetes patients. However the effect on C-peptide level was not accompanied by a reduction in insulin usage or lower HbA1c levels (Raz et al. 2001, 2007). A phase III study in adults is currently ongoing while no DiaPep277 effect has been reported in young children (Lazar et al. 2007).

39.1.3 Short Bowel Syndrome

Short bowel syndrome (SBS) occurs after an extensive loss of small intestinal length, typically after surgery, which leads to a malabsorption of fluid and nutrients. In a timespan of about 24 months following surgical resection of small intestinal segments, the remaining intestine undergoes adaptation through several mechanisms which aim at increasing the absorptive capacity, a process which has been recognized first 100 years ago (Flint 1912). These include villous cell hyperplasia, increased crypt depth, intestinal dilatation, increased mucosal enzyme activity and reduction of intestinal transit (Nightingale and Lennard-Jones 1993). The underlying mechanisms were shown to involve growth factor and specific nutrients, such as growth hormone, insulin-like growth factor 1, glucagon-like peptide 2, glutamine, short chain fatty acids and pancreatic-biliary secretions (Tamada et al. 1993; Jacobs 1983; Seguy et al. 2003; Ellegard et al. 1997).

Nevertheless, a subset of patients will develop intestinal failure, meaning the inability to maintain an adequate balance of nutrients and water even after the postoperative adaptation phase, and suffer from dehydration and malnutrition without dietary support. In particular, a residual small bowel length of less than 100 cm leading to an end stoma or less than 50 cm connected to a functioning colon poses a risk factor for the need of long-term parenteral nutrition or, in a selected number of patients, small bowel transplantation. Parenteral nutrition (PN) is the therapy of choice for intestinal failure, but carries considerable risks such as hepatic failure, central vein thrombosis, recurrent infections, and a reduced life expectancy. However, it is still superior to small bowel transplantation, which is encumbered by high incidences of graft rejection and other postoperative complications (Pironi et al. 2008, 2011).

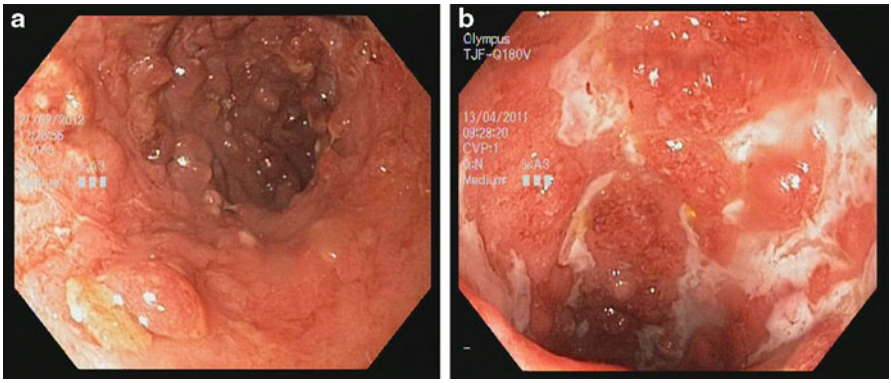


Fig. 39.1 Endoscopic aspect of ulcerative colitis (**a**) with multiple pseudopolyps and Crohn's disease (**b**) with severe longitudinal ulceration

Supportive medical management is directed to reduce stool output to <2 L per day. It includes agents that reduce secretion, such as proton pump inhibitors and octreotide, and motility, such as loperamide and opium, but also dietary advice which meets the requirements of the postoperative anatomy.

39.1.4 Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract, including Crohn's disease (CD) and ulcerative colitis (UC). They are characterized by recurrent mucosal inflammation and ulceration (Fig. 39.1), leading to various intestinal and extra-intestinal manifestations. Crohn's disease mostly involves the distal ileum and/or the colon, whereas Colitis ulcerosa is restricted to the colon. The pathogenetic mechanisms that cause the two types of inflammatory bowel disease are still under investigation. It has originally been suggested that they develop in a genetically predisposed subject due to a dysregulated adaptive immune response to unknown antigens, resulting in continuous immune mediated inflammation (Ardizzone and Bianchi 2002; Fiocchi 1997). Currently, there is broad agreement that luminal microbes are playing an important role in the development of IBD, since both disease locations are characterized by high concentrations of intestinal bacteria, and the adaptive immune response is directed against the microbiota. Increasing evidence has shown that defects in the innate immunity are at the centre of both types of IBD. In healthy mucosa, an adequate secretion of antimicrobial peptides and the mucus layer act as a barrier against microbes. It was shown in the last years that the differentiation from the intestinal stem cell towards the Paneth cell in ileal CD and the goblet cell in UC might be impaired, which leads to a defective antimicrobial barrier and thus, microbes can invade the mucosa and cause inflammation (Gersemann et al. 2012).

Current treatment procedures for CD and UC variably affect the inflammatory events, and indeed no available drug is at present curative. Therapy is often implemented stepwise through aminosalicylates, antibiotics, corticosteroids, immunosuppressive medications including thioguanine compounds, methotrexate, ciclosporin, and finally anti-TNF drugs. Many patients require surgery to combat complications. In the future, patients may benefit from new therapeutic approaches stimulating the protective innate immune system.

39.2 Medical Regenerative Therapies for Type 1 Diabetes and Intestinal Disease

39.2.1 Beta Cell Regenerative Strategies for Type 1 Diabetes

The results from islet cell transplantation demonstrated that diabetes can be cured by replenishing the beta-cell mass. As a result treatment strategies have been developed aimed at restoring beta cell mass and function such as stimulation of insulin secretion and islet neogenesis. However it must be emphasized that based on preclinical and clinical results a combination of both immunomodulatory and regenerative strategies could greatly improve clinical outcome.

39.2.1.1 Stimulation of Beta Cell Function

Incretin hormones, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) function by regulating after meal blood glucose levels via mechanisms including enhancement of glucose–stimulated insulin release and reduction of postprandial glucagon levels (Drucker 2003). In murine models, GLP-1 and its analog exendin-4 were also reported to function by increasing beta-cell replication, decreasing beta-cell apoptosis, stimulating beta-cell neogenesis, and inducing beta-cell expansion (Xu et al. 1999; Farilla et al. 2003; Li et al. 2005). Combination therapy trials using either GPL-1 and anti-lymphocyte serum (ALS) or exendin-4 and anti-CD3 resulted in disease reversal in overt diabetic and recent onset NOD mice, respectively. Treated mice showed an increase in insulin secretion with no effect on beta cell replication or beta cell apoptosis (Ogawa et al. 2004; Sherry et al. 2007). In type 2 diabetes patients, GLP-1 and exendin-4 have been shown to stimulate insulin secretion in remaining beta cells (Kolterman et al. 2003; Buse et al. 2004). However clinical trials for type 1 diabetes conducted with a combination of exenatide (a synthetic version of exendin-4) and daclizumab (an immunosuppressive anti-CD25 monoclonal antibody) yielded disappointing results: Exenatide resulted in delayed gastric emptying, suppressed endogenous incretin levels, but did not increase C-peptide secretion in most trials (Rother et al. 2009). Besides this, β-cell replication in humans is more difficult to stimulate compared to rodents.

39.2.1.2 Beta Cell Neogenesis

The beta cell mass is dynamic and undergoes expansion and contraction depending on metabolic needs (e.g. during normal growth, pregnancy, obesity) (Bonner-Weir 1994). Mechanisms such as replication of pre-existing beta-cells or the formation of new beta cells from progenitor cells (neogenesis) have been associated with beta cell mass expansion. The exact contribution of these two mechanisms to beta cell mass regeneration is still under debate. However, differences in the balance of these pathways have been shown to be species- and age-dependent. Murine beta cell expansion dynamics differ from human dynamics, with β-cell replication being predominant in murine models, whereas neogenesis was more evident in type 2 diabetes patients and non-diabetic individuals (Bonner-Weir et al. 2010; Bouwens and Pipeleers 1998; Butler et al. 2003a, b; Dor et al. 2004).

Murine models of injury such as partial pancreatectomy and partial duct ligation (PDL) have been used to study beta cell neogenesis (Bonner-Weir et al. 1993; Wang et al. 1995). Beta cell neogenesis occurs either via stem/progenitor cell activation and/or transdifferentiation. The identity of the stem/progenitor cell for neogenesis is however still elusive but the PDL model demonstrated that regeneration is limited to the portion distal to the site of ligation. Furthermore, neurogenin 3 (a transcription factor involved in development of pancreatic endocrine cells), was induced in cells in or adjacent to the pancreatic ducts after PDL. These neurogenin 3+ cells yielded islets including beta cells (Xu et al. 2008). These studies hence suggest that beta cells can be formed from progenitor cells within the pancreatic duct epithelium. Conversely, administration of the beta-cell toxin alloxan prior to PDL resulted in the generation of new beta-cells from adult alpha cells (Chung et al. 2010), thereby suggesting that alpha cells of the islets of Langerhans may be a source of beta cell progenitors.

Taken together, these studies show that beta-cell neogenesis is feasible *in vivo*; therefore, induction of beta cell neogenesis could be an excellent way to restore beta-cell mass for effective T1D treatment. Hormones and growth factors have been shown to induce beta-cell neogenesis (Wang et al. 1993; Rooman et al. 2002; Rooman and Bouwens 2004). However, any form of beta cell replacement in T1D will also need induction of immune tolerance to prevent the destruction of cells by the autoimmune response.

Gastrin and Epidermal Growth Factor

Infusions of the peptide hormone gastrin in a rodent PDL model induced neogenesis and expansion of beta-mass from transdiffentiated exocrine pancreas. These studies showed an increase in beta cell mass in the ligated portion of the pancreas that was not associated with increased proliferation and hypertrophy or reduced beta-cell death (Rooman et al. 2002). Furthermore a combination of gastrin and epidermal growth factor (EGF) was shown to restore normoglycemia, increase beta-cell mass, density and pancreatic insulin content in alloxan treated mice. Monotherapy with either

hormone was reported to have no effect on hyperglycemia (Rooman and Bouwens 2004). The effect of gastrin and EGF was further confirmed in NOD mice as treated recent onset diabetic NOD mice exhibited increased beta cell mass and reversal of hyperglycemia (Suarez-Pinzon et al. 2005). Furthermore combination therapy with gastrin and GLP-1 resulted in reversal of hyperglycemia, downregulation of autoimmune response and protection of beta cells from apoptosis in NOD mice (Suarez-Pinzon et al. 2008). Gastrin and EGF therapies have been translated into clinical trials with results from a phase II clinical trial conducted with an EGF analog E1-1NT showing a 35–75% reduction in insulin usage and maintenance of blood glucose levels in some T1D patients. Currently, a phase I combination trial with EGF and gastrin is ongoing (van Belle et al. 2011). In parallel a phase II trial is being performed using gastric proton pump inhibition to increase endogenous gastrin in combination with a GLP-1 analogue.

Islet Neogenesis Associated Protein (INGAP)

Islet neogenesis associated protein (INGAP) is a member of the regenerating gene (Reg) family of proteins. The Reg family is part of the C-type lectin super family and is mainly involved in the proliferation or differentiation of liver, pancreas, gastric and intestinal cells (Zhang et al. 2003). INGAP is believed to be the initiator of neogenesis in particular the INGAP104-118 peptide. This peptide stimulates an increase in beta cell mass in mice, rats, hamsters and dogs (Lipsett et al. 2007). INGAP has been found to be overexpressed in islets from patients with recent onset-type1 diabetes, and administration of INGAP into streptozotocin induced diabetic mice resulted in reversal of disease and an increase in beta cell mass (Rosenberg et al. 2004). Phase I and II trials with INGAP showed an increase in C-peptide secretion, improved glycemic control but no decrease in HbA1c levels. A trial is ongoing to optimize dosing, exposure, formulations and possible combination therapies (Dungan et al. 2009).

39.2.2 Growth Hormone and Glutamine in Short Bowel Syndrome

Growth hormone exerts its trophic effects on the intestine via IGF-1, which originates from lamina propria mesenchymal stem cells. The resulting increase in DNA and protein production involves ornithine decarboxylase activity, for which glutamine is a substrate. Previous animal studies have shown that growth hormone and glutamine both have beneficial effects on intestinal adaptation in the early phase after surgery (Gouttebel et al. 1992) and act synergistically on intestinal function (Gu et al. 2001). Subsequently, further basic research could document the benefit of growth hormone and glutamine in human intestine (Scheppach et al. 1994; Inoue et al. 1994). Byrne and colleagues first demonstrated 15 years ago the efficacy of

growth hormone and glutamine in promoting intestinal adaptation in an open-label clinical trial and a case series (Byrne et al. 1995a, b) with SBS patients. These results attracted much interest, and several randomized controlled trials were carried out in the following years, which were also the topic of a Cochrane review in 2010 (Wales et al. 2010).

The clinical studies examining the effects of growth hormone with or without glutamine demonstrated an increase in weight, lean body mass and absorptive capacities, but the benefit was short-lived after therapy cessation (Byrne et al. 1995a; Ellegard et al. 1997; Scolapio et al. 1997; Seguy et al. 2003; Jeppesen et al. 2001). Only one study was able to document a sustained effect on PN volume, calories and infusion number in growth hormone and glutamine-treated patients at the 3 months follow-up (Byrne et al. 1995a). Analysis of the fat- and energy absorption yielded heterogeneous results, which is likely due to the different outcome measures and differences in patient selection (e. g. with or without underlying mucosal disease, age, or nutritional status). Only two of the trials found a positive effect on fat absorption at the end of treatment (Jeppesen et al. 2001; Seguy et al. 2003). Furthermore, the dose and therapy duration may explain the differences between the clinical trials. While a lower dose of growth hormone led to an increase in absorption in one trial (Seguy et al. 2003), higher doses in two different trials did not. This may be partially explained by observations from animal studies, where an excess of growth hormone caused a reduced, possibly compensatory, responsiveness of crypt proliferation to growth factor signaling (Lund 1998; Dahly et al. 2004). The question whether glutamine addition to growth hormone treatment further enhances the clinical benefit is still controversial; in the studies using both compounds (Byrne et al. 1995a; Scolapio et al. 1997; Jeppesen et al. 2001), neither the crude results nor the subgroup analyses from the Cochrane review (Wales et al. 2010) detected significant differences.

Overall, the data is still insufficient to routinely recommend growth hormone treatment for SBS. There is some benefit in terms of weight gain and fat absorption, but the patient numbers are very small, and the effects short lived. Evidence regarding long-term safety is non-existent. Furthermore, the question whether glutamine addition is beneficial remains unresolved. Growth hormone treatment may not be justified when considering benefit and costs.

39.2.3 Intestinal Growth Factors in Inflammatory Bowel Disease

IBD treatment has largely focused on decreasing inflammation. Given a component of dysfunctional epithelial repair, several studies have investigated the effect of intestinal growth factors to treat not only growth retardation in pediatric IBD, but also inflammation in children and adults. In a preliminary study, 37 patients with moderately to severely active Crohn's disease were treated with subcutaneous growth hormone vs. placebo and were instructed to increase their protein intake. At 4 months, CDAI had decreased by a mean of 144 points in the treatment group,

and by only 19 points in the placebo group, which represented a statistically significant difference. In terms of side effects, several patients experienced headache and edema. The authors concluded that growth hormone treatment can be beneficial in active CD (Slonim et al. 2000). In a pediatric study, 20 patients were treated with systemic corticosteroids plus either growth hormone or placebo. Remission rates after 12 weeks of treatment were 65% in the combined treatment group vs. 20% in the monotherapy group. While the addition of growth hormone produced a positive effect on growth failure, the intestinal mucosal inflammatory state as assessed by endoscopy was not different between the groups (Denson et al. 2010).

Epidermal growth factor can induce epithelial growth by activation of PI3-kinase, AKT and MAPK pathways. Based on concerns that systemic EGF may induce epithelial neoplasia, one study investigated the effect of recombinant EGF enema (5 μg) vs. placebo (n = 12 patients in each group) in mild-to-moderate ulcerative colitis. Disease remission defined as a St. Mark's score <4 was achieved by 10 patients in the EGF group and 2 patients in the placebo group. However, both groups received additional mesalamine, which may have biased the results towards a more positive outcome, considering that a EGF-only group was not included (Sinha et al. 2003).

Controlled clinical trials with keratinocyte growth factor (Sandborn et al. 2003), trefoil factor (Playford et al. 1996), GMCSF (Dieckgraefe and Korzenik 2002), and sargramostim (Korzenik et al. 2005) did not reveal a benefit over placebo. Although the approach to use intestinal growth factors to treat inflammatory bowel disease, possibly as an adjunct to anti-inflammatory therapy, is intriguing and may aid the restitution of the damaged mucosa, none of the above therapies can today be recommended for widespread use, and concerns regarding their potential to induce neoplastic growth remain.

39.2.4 Targeting GLP-2 Signaling to Treat Intestinal Diseases

The 33 amino acid peptide GLP-2 is a key mediator of intestinal adaptation (Scott et al. 1998). It is secreted from neuroendocrine cells and increases absorptive capacity by augmenting crypt cell proliferation and reducing villous cell apoptosis (Drucker et al. 1996). Additionally, it inhibits gastric emptying and acid secretion, reduces intestinal permeability, and modulates inflammatory responses and mesenteric blood flow (for review see Tee et al. 2011). Its half-life is very short, since it undergoes N-terminal truncation by the proteolytic enzyme dipeptidyl peptidase IV.

G-protein coupled GLP-2 receptors are expressed on enteroendocrine cells, enteric neurons, and subepithelial fibroblasts of the small intestine (Guan et al. 2006; Yusta et al. 2000; Orskov et al. 2005). A variety of effectors appear to be involved in mediating the downstream signal, including insulin-like growth factor-1 for epithelial proliferation, nitric oxide for the upregulation of intestinal blood flow, vascular endothelial growth factor and transforming growth factor-β for wound repair, and vasoactive intestinal peptide for anti-inflammatory effects (Rowland and Brubaker 2011) (Fig. 39.2).

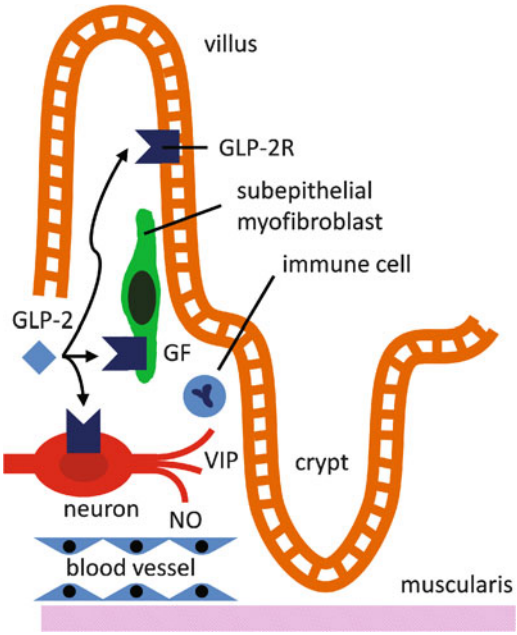


Fig. 39.2 GLP-2 action in the intestine. GLP-2 receptor (GLP-2R) is expressed on endocrine cells, subepithelial myofibroblasts and neurons. The effects of GLP-2 on epithelial proliferation, inflammation and blood flow are probably conferred to a large part in an indirect manner, involving nitric oxide (NO), vasoactive intestinal polypeptide (VIP), and growth factors (GF) such as IGF-1

Teduglutide is a DPPVI-resistant analog of GLP-2 lacking the N-terminal cleavage site to extend its half-life. Initially, an open label study of SBS patients demonstrated an increase in wet-weight absorption of up to 1 L daily without a significant effect on energy absorption, which was maintained for 24 months of treatment, but was quickly reversed upon treatment discontinuation (Jeppesen et al. 2005). Subsequently, a multinational, randomized, placebo-controlled study involving 83 patients suffering from SBS in need of parenteral nutrition at least three times a week for 1 year or more was carried out (Jeppesen et al. 2011). After a 4–8 week stabilization period to allow for optimization of parenteral nutrition, patients were randomized to receive either placebo, 0.05 mg/kg/day teduglutide, or 0.1 mg/kg/day teduglutide for 24 weeks. The primary endpoint was a graded response score accounting for the intensity and the duration of the response, which was defined as a reduction of the requirement for parenteral nutrition by 20%, with urine production being used as a surrogate marker. While the graded response score as well as a 20% reduction of parenteral nutrition were achieved by a significantly higher proportion of patients in the 0.05 mg/kg/day group than in the placebo group (16/35 [46%] vs. 1/16 [6%]), the difference of the 0.1 mg/kg/day arm vs. placebo was not significant. The latter result was explained by group differences and the fact that the high-dose group displayed a significant reduction in oral fluid intake. Additionally, there was a small increase in body weight despite reduced calorie provision via parenteral nutrition, and a positive effect on trophic markers of the intestine. A follow-up study with the subjects completing this trial was published in abstract form (Gilroy et al. 2008). Patients previously receiving active treatment were maintained

on their dose, and patients previously treated with placebo were randomized to treatment with 0.05 or 0.1 mg/kg/day teduglutide. In both groups, 75% of the subjects were able to maintain their PN reduction. In a confirmatory phase 3 study (STEPS – study of teduglutide in PN-dependent short bowel syndrome, NPS pharmaceuticals press release), 86 patients were treated with either 0.05 mg/kg/day active drug or placebo for 24 weeks to investigate whether a 20–100% reduction in weekly PN volume at weeks 20 and 24 was feasible. 63% of teduglutide-treated vs. 30% of placebo-treated patients reached this endpoint. In addition, patients in the teduglutide group were able to reduce the weekly PN volume from 12.9 to 8.5 L, which was also significant vs. placebo. A 2-year follow-up study (STEPS 2) is underway.

Most studies with GLP-2 and teduglutide have not shown a significant increase of adverse events. Abdominal complaints, headache, and injection site reactions were commonly reported. In patients with congestive heart failure or a history of bowel obstruction, these therapies should be administered with caution. A remaining concern arises from the proliferative properties of GLP-2 agonists and therefore their potential ability to induce malignancies. This is of particular importance, since treatment of SBS with teduglutide may be life-long. In azoxymethane-treated mice, GLP-2 indeed had pro-carcinogenic effects (Iakoubov et al. 2009). Until the safety profile with long-term treatment has been clearly established in clinical studies, thorough screening for premalignant lesions, such as colonic adenomas, is advisable.

Based on the positive clinical data, teduglutide has been granted orphan drug status by the FDA and EMEA, and a new drug application for this first-in-class SBS treatment has been filed to the FDA in the fourth quarter of 2011. In summary, trials with teduglutide in SBS have shown encouraging results, making this drug a new therapeutic option for SBS with the potential to reduce PN dependence and complications. Future studies will have to delineate the long-term outcomes, safety, and optimal therapeutic regimen.

Since it has been shown that GLP-2 can act anti-inflammatory and promote epithelial repair, studies have been carried out to investigate the effect of manipulating GLP-2 signaling in Crohn's disease. In an 8-week, controlled pilot study, 100 patients were treated with teduglutide, and their CDAI determined (Buchman et al. 2010). The higher teduglutide dose led to remission rates of 55.6% vs. 33.3% (placebo). This difference was not statistically significant, and secondary outcomes like surrogate markers of inflammation, were not reported.

39.3 Cell-Based Therapies

39.3.1 Stem Cells Approaches for Diabetes Therapy

Stems cells have been reported to hold great promise for T1D therapy due to their immunomodulatory role and regenerative capacities. Furthermore, drawbacks encountered in the optimization of islet cell transplantation have prompted researchers to search for other potential sources of glucose producing tissues including stem cells.

Stem cells are defined by their ability to self-renew and to differentiate into many specialized cell types, tissues or organs. Stem cells can be classified as either pluripotent (with the ability to differentiate into all cell types) or multipotent (with a limited differentiation capacity) (Tuch et al. 2011).

39.3.1.1 Pluripotent Stems

Embryonic Stem Cells (ESC)

ESCs are derived from the inner cell mass of a blastocyst. These cells express the transcription factors Oct-4, Nanog-1 and Sox2, which are involved in self-renewal and act as markers of pluripotency (Friel et al. 2005). ESCs are able to differentiate into the ectoderm, mesoderm, and endoderm (from which the pancreas is derived). The rationale behind the use of ESCs for T1D therapy is that under certain conditions, these cells can be steered to differentiate into pancreatic islet cells that can in turn be transplanted into patients.

Various ESC differentiation protocols have been developed based on the development of the embryonic pancreas. Many of the earlier attempts to generate functional islets *in vitro* from ESCs were limited by final cell homogeneity, immaturity of differentiated cells, low numbers of insulin producing cells, and poor insulin responses to glucose exposure (McCall et al. 2009). However, a recent study from Baetge and colleagues showed that implantation of pancreatic endoderm derived from human ESCs (hESCs) into mice could efficiently generate glucose-responsive endocrine cells after implantation. These results imply the need for *in vivo* differentiation in order to generate glucose responsive cells. Furthermore, these cells could protect against streptozotocin-induced hyperglycemia (Kroon et al. 2008). Conversely, a separate study conducted by implanting hESCs derived pancreatic endoderm into athymic nude rats confirmed the development of islet-like structures but upon glucose challenge no increase in C-peptide or insulin was observed. These results led the researchers to conclude that though islet-like structures were formed from implanted hESC differentiated pancreatic endoderm, the extent of endocrine cell formation and secretory function is not yet sufficient to be clinically relevant (Matveyenko et al. 2010). Apart from the complexities of generating functional islets, ESCs use is also limited due to ethical concerns. Furthermore, since ESCs are developed from an allogeneic donor, strategies need to be developed to protect the ESCs from immune attack. Moreover, ESC cells have been shown to give rise to teratomas and teratocarcinomas in humans (Guleria et al. 2007).

Induced Pluripotent Stem Cells (iPs)

A major breakthrough in the pluripotent stem cell field was the development of induced pluripotent stem cells (iPS). iPS cells are derived from somatic cells in which pluripotency is restored by the induced expression of the transcription factors

Oct-4, Sox2, Nanog, c-Myc, LIN28, and Klf4 (Stefanovic et al. 2009). With regards to morphology, self-renewal capacity, and differentiation iPS and ESC cells are very much alike; however, iPS cells have an added advantage over ESC cells in that they allow for the possibility of autologous cell therapy. Indeed, Maehr and colleagues showed for the first time that it was possible to generate iPS cells from dermal fibroblasts of T1D patients. This process involved retroviral transduction of the fibroblast with Oct4, Sox2, and Klf4. Furthermore, these iPS cells showed a normal karyotype and beta-cells derived from these iPS cells were shown to be C-peptide positive and were capable of releasing insulin after *in vitro* stimulation with glucose (Maehr 2011). However, a lot of research still needs to be done before iPS cells can be considered for clinical trials. Furthermore, iPS cells are also limited by the formation of teratomas, and the use of retroviral vectors for delivery of reprogramming factors could further lead to malignant transformation.

39.3.1.2 Multipotent Stem Cells

Hematopoietic Stem Cells (HSCs)

Adult stem cells/multipotent stem cells can be classified into hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs are by far the best characterized and best studied stem population. HSCs are located in the bone marrow niche and can be readily harvested from bone marrow and umbilical cord blood (UCB). HSCs can also be collected from peripheral blood after mobilization from bone marrow with granulocyte colonystimulating factor (G-CSF) (Brignier and Gewirtz 2010; Flomenberg et al. 2005). Additionally HSCs can be isolated on the basis of their surface markers, that is, lineage specific antigen negative (lin-), CD34,+ CD38,– CD133,+ c-Kit/CD117,+ CD59,+ Thy1/CD90+, and CXCR4+ cells. HSCs preferentially differentiate into lymphoid and myeloid lineages.

The rationale behind the use of HSC transplantation (HSCT) for the treatment of autoimmunity is that transient lymphoablation followed by autologous HSCT will allow for immune regeneration and resetting of immune self-tolerance (Couzin-Frankel 2010). A clinical trial for T1D was conducted in Brazil using high dose cyclophosphamide plus rabbit polyclonal anti thymocyte globulin (ATG) for lymphoablation followed by an autologous HSCT. Analysis of this study showed an increase in C-peptide levels, good glycemic control, and insulin independence in a majority of the treated patients (20 out of 23 recent onset diabetics). Furthermore, 12 of these patients were insulin independent for 31 months whilst the other 8 patients had periods ranging from 6 to 47 months when they were insulin free after which they resumed insulin therapy albeit at a lower dose than pretransplant (Voltarelli et al. 2007; Couri et al. 2009). Additionally, using the same HSCT protocol in eight recent onset patients, Snarkski and colleagues achieved insulin independence in all the patients, except one who resumed low dosage insulin treatment 7 months after transplantation (Snarski et al. 2011). Although these results seem promising, this treatment was associated with side effects such as nausea, vomiting, fever and

alopecia. Two patients presented with nosocomial pneumonia and cases of Grave's disease, transient hypergonadotropic hypogonadism, and autoimmune hypothyroidism were reported. However, no mortality was observed so far (Couri and Voltarelli 2009). Long-term consequence of the conditioning regimen need to be monitored, and it remains questionable how much risk of immune interventions we dare to take for a disease which can safely be treated with insulin replacement for decades. Taken together, the studies involving HSCs transplantation have taught us what can be achieved with appropriate immune therapies.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a heterogeneous population of multipotent stem cells that can differentiate into various mesodermal cell lineages including myocytes, osteoblasts, chondroblasts, fibroblasts and adipocytes. MSCs can be found in almost every organ, but for therapeutic use they are isolated from the UCB and bone marrow. Human MSCs can be identified by their lack of HSC markers, their expression of CD105, CD73 and CD90 and ability to adhere to plastic. MSCs possess anti-inflammatory and immunomodulatory properties mediated by the secretion of factors such as indolamine 2,3-dioxygenase, IL-6, TGF-β1, inducible nitric oxide synthetase and prostaglandin (Brignier and Gewirtz 2010). Furthermore, MSCs have been shown to induce regulatory T cells and to suppress effector and cytotoxic T cells and B cells *in vitro* (Selmani et al. 2008). The immunomodulatory effects of MSCs are still under exploration for their application in T1D therapy. However, transfer of MSCs into prediabetic NOD mice was shown to result in disease prevention and induction of IL-10 secreting FOXP3+ regulatory T cells (Madec et al. 2009). Furthermore, transfer of human MSCs (hMSCs) into a streptozotocin induced diabetes model resulted in decreased hyperglycemia associated with an increase in beta cells mass and insulin production. In addition, it was observed that the hMSCs selectively homed to both pancreatic islets and renal glomeruli of the diabetic mice leading to tissue repair. This suggests that hMSCs may be useful in enhancing insulin secretion and perhaps improving the renal lesions that develop in patients with diabetes mellitus (Lee et al. 2006).

MSCs have also been reported to be a potential alternative source for β-cell neogenesis. Retroviral transduction of the MSCs with the β-cell development transcription factor pancreatic and duodenal homeobox 1 (Pdx1), followed by *in vitro* steering in the presence of islet hormones resulted in the generation of insulin producing cells. Transplantation of these cells under the kidney capsule resulted in decreased hyperglycemia in a streptozotocin induced diabetes model. Furthermore, insulin producing cells could be generated from diabetic patients without genetic manipulation thereby demonstrating that long-term hyperglycemia does not constitute a factor against iPS generation in T1D patients (Li et al. 2007; Sun et al. 2007). Phase I/II clinical trials aimed at assessing the efficacy and safety of allogeneic or autologous MSCs therapy for T1D are currently ongoing (Fiorina et al. 2011).

39.3.1.3 Combination Therapy: The Future of T1D Treatment

Most of the clinical trials discussed thus far have been largely based on monotherapies which were either aimed at tolerance induction and beta cell preservation, or restoration of beta cell mass and function. However, many researchers in the field of T1D argue that like in cancer, T1D treatment could benefit from combination therapy thereby resulting in tolerance induction and beta cell regeneration (Bresson and von Herrath 2007). They propose combinations of treatments with either a documented effect as a monotherapy in T1D or a combination treatment that has been efficacious in another autoimmune disease (van Belle et al. 2011). Combination therapies have been largely successful in preclinical rodent models. For example, studies in BB rats showed disease delay or prevention after administration of the purine biosynthesis inhibitor mycophenolate mofetil (MMF) and anti-CD25 mAb (Ugrasbul et al. 2008). Furthermore, intranasal insulin in combination with anti-CD3 mAb was shown to be more efficacious than the monotherapies at reversing recent onset diabetes in NOD mice and a RIP-LCMV diabetes model. This treatment resulted in the expansion of CD25+Foxp3+ and IL-10, TGF-β and IL-4 insulin-specific Tregs, and these regulatory T cells could confer dominant tolerance to immunocompetent recent onset diabetic recipients (Bresson et al. 2006). These results suggest combination therapy with immune modulators and islet antigen specific vaccines could be effective. Additionally based on the success of anti-CD3 mAb in combination with exendin-4 treatment in recent onset diabetic NOD mice, combination treatments with immune modulators and compounds that enhance β-cell mass and function have also been proposed for future clinical trials (van Belle et al. 2011; Sherry et al. 2007). This approach is postulated to have a higher success rate than the gastrin and exenatide β-cell regeneration trials owing to the fact that anti-CD3 induces tolerance whilst exenatide increases insulin secretion from residual β-cells. Clinical trials so far have shown that intensive insulin therapy in combination with exenatide and anti-CD25 mAb showed no improved function of remaining β-cells in patients with long-standing diabetes (van Belle et al. 2011; Rother et al. 2009). However, these results do not rule out the efficacy of combination therapy in recent onset diabetes patients.

The aforementioned anti-CD3 and intranasal insulin preclinical study demonstrated that adoptive transfer of regulatory T cells results in reversal of disease in recent onset diabetic mice (Bresson et al. 2006). The efficacy of adoptive transfer of CD4+CD25+Foxp3+ natural Tregs (nTregs) in human trials has been demonstrated in the treatment of leukemia by hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusions (DLI). Adoptive transfer of *ex vivo* purified nTregs in this setting was shown to be safe and the transferred nTregs promoted lymphoid reconstitution, did not overtly weaken the graft vs. leukemia effect, and prevented graft versus host disease (Edinger and Hoffmann 2011). These findings open up the possibility of nTreg transfer as a potential immunomodulatory approach for T1D patients. The HSCT trials mentioned above utilized polyclonal nTregs however adoptive transfer of antigen specific nTregs was shown to be more effective at disease prevention and reversal in recent onset and overtly diabetic NOD mice

(Katz et al. 1993; Salomon et al. 2000; Wu et al. 2002; Tang et al. 2004; Tarbell et al. 2006; Masteller et al. 2005; Jaeckel et al. 2008). The frequency of antigen specific nTregs coming from a polyclonal repertoire is very low however the successful treatment of colitis in a murine model with nTregs redirected by antigen-specific chimeric receptor gives hope for the potential use of nTregs with redirected specificities for T1D treatment (Elinav et al. 2009). Furthermore, the fact that T cells with redirected specificities are currently in use for a plethora of clinical trials for cancer (Jena et al. 2010; Morgan et al. 2010) and nTreg transfer was shown to be safe in HSTC implies that adoptive transfer of redirected nTregs could also be an acceptable therapy in T1D patients. Furthermore, one could envision combination therapies of adoptive transfer of redirected Tregs with compounds that enhance beta cell mass and compounds that favor *in vivo* Treg expansion such as rapamycin (Battaglia et al. 2005).

39.3.2 Stem Cell Approaches in the Therapy of Intestinal Disease

Experimental and clinical research increasingly utilizes stem cell therapy for IBD. Initially, the excitement surrounding the stem cell field was based on the unique biological properties of these cells and their capacity to self-renew and regenerate tissue and organ systems. Later on, the immunomodulatory ability of stem cell therapy has become apparent. Conventionally, the definition of stem cells refers to the hematopoietic stem cells (HSC), with reference to myeloid and lymphoid lineages. However, a distinct lineage is now known to consist of mesenchymal stem (stromal) cells (MSC). Stem cell therapy for IBD is thought to both repair damaged intestinal tissue and the immune system. For active luminal disease three forms of stem cell therapy have been attempted. First, bone marrow derived donor (BMD) or autologous stem cells (bone marrow contains both hematopoietic as well as mesenchymal stem cells), second, peripheral blood donor or autologous stem cells and third, donor or autologous mesenchymal stem cells from adipose tissue. Autologous and donor stem cell transplantation has involved pre-transplantation bone marrow ablation, while mesenchymal stem cell studies have avoided bone marrow ablation (Table 39.2).

39.3.2.1 Hematopoietic Stem Cells

The possibility that HSCT might be an effective treatment in CD arose from a case report published in 1993, reporting a female patient with CD who remained symtom-free during a 6 month follow-up after HSCT for non-Hodgkin's lymphoma (Drakos et al. 1993). This report was followed by several other case reports and a case series in 1996. In this case series, remission was observed in four out a five patients with CD and leukemia after allogenic bone marrow transplantation

Table 39.2 Types of stem cells for treatment in IBD

	Hematopoietic	Mesenchymal	Intestinal
Origin	bone marrow, peripheral blood	bone marrow, adipose, placenta	intestinal crypt
Function	>Autologous or allogeneic hematopoietic stem cell transplantation (HSCT) >Elimination of auto-reactive lymphocytes (lymphoablative effect) >Altered immune reconstitution after immuneablation >Generation of naive cells to restore tolerance	>Autologous or allogeneic transplantation >Antiproliferative for stimulated T-cells >Inhibits inflammatory response of innate and adaptive immune system (immunomodulatory effect) >Reparative effect on inflamed tissue >Adipose derived stem cells for treatment of fistulizing diseases	>Keeps intestinal barrier functional >Keeps crypt sterile through secretion of antimicrobial peptides and defensins >Genetic repair approach in future might supply defective genes through genetic transfer techniques

(Lopez-Cubero et al. 1998). Five of these six patients had active CD, and four of the five patients had sustained remission of CD 54–183 months after transplantation. In another case series, remission of symptoms occurred in all patients (six with CD, four with UC) after myeoloablative treatment and allogeneic bone marrow transplantation (Ditschkowski et al. 2003). In this study, one patient had a mild self-limiting recurrence and another died of infectious complications. All patients except two maintained immunosuppressive therapy at the end of follow-up (follow-up 3–117 months). Although these studies were not designed to investigate the role of HSCT on IBD, they supported the notion that lymphoablation and generation of new self-tolerant lymphocytes might induce remission in patients with IBD.

Based on these results, attempts with HSC as a primary treatment were initiated. The first case was reported in 2003 (Craig et al. 2003). These initial trials are integrated in the final report of a phase I study in which infusion of autologous HSCs from peripheral blood after mobilization, expansion and conditioning in 12 patients with refractory CD was performed (Oyama et al. 2005). Eleven patients had remission after 6 months, monitored by Crohn's Disease Activity Index (CDAI) <150, and after 18.5 months of follow-up, only one patient has experienced recurrence. Another trial used peripheral HSC unselected for CD34 in four patients successfully (mean follow-up 16.5 months) (Cassinotti et al. 2008). In a long term follow-up of 24 patients by the group from Chicago, the percentage of clinical relapse-free survival, defined as the percentage of patients restarting medical therapy after transplantation, was 91% in 1 year, 63% at 2 years, 57% at 3 years, 39% at 4 years and 19% at 5 years, showing that 81% of these patients had to begin medical therapy again 5 years after transplantation (Burt et al. 2010).

The sustained clinical remission with hematopoietic stem cell therapy seems not only to be due to cyclophosphamide and G-CSF during mobilization (Kreisel et al. 2003). The mechanisms underlying the beneficial effects remain unclear, but are probably the result of initial eradication of T-cells and memory cells because of the lymphoablative effects of drugs used in the conditioning regimen. Later on, there may be an effect of altered immune reconstitution. In 2005, the international committee established for the development of guidelines on entry criteria and transplant protocols for IMIDs (immune mediated inflammatory diseases) recommended that autologous HSCT should be preferred to allogeneic HSCT because of a lower risk of severe toxicity (Gratwohl et al. 2005). It has to be kept in mind that stem cell collection and autologous transplantation are associated with morbidity and mortality, and that flares of the disease and lethal complications during mobilization have been reported (Kapoor et al. 2007).

In a study based on genetic linkage analysis and candidate-gene sequencing on samples from two unrelated consanguineous families with children with early-onset inflammatory bowel disease, three distinct homozygous mutations in genes IL10RA and IL10RB were identified that segregated with disease phenotype (Glocker et al. 2009). One member of the family with IL10RB mutation suffered from a severe CD since his third month of life with proctitis, abscesses and multiple surgical interventions. This subject underwent allogeneic HSCT using an HLA matched sibling not carrying the mutation. Fistulas resolved shortly after transplantation, and the patient remained in continuous remission from ileocolitis during a follow-up period of 2 years after transplantation. This was the first study showing a curative approach to severe CD by means of allogeneic HSCT that was justified by a monogenic cause of the disease in this case.

Currently, there are two trials active but not recruiting on stem cell transplantation in patients with Crohn's disease (NCT00271942, NCT 00278577). A phase III randomized and controlled trial is now recruiting patients in Europe to address the individual steps of the autologous peripheral blood stem cells transplantation (aPBSCT) protocol to treat CD (ASTIC, NCT00297193). Another trial addresses the efficacy of aPBSCT in pediatric patients (NCT00692939), and a third uncontrolled trial is recruiting to assess the efficacy of allogeneic PBSCT to treat CD (NCT01288053).

39.3.2.2 Mesenchymal Stem Cells

When cells from a bone marrow aspirate are cultured in plastic flasks, hematopoietic cells and stem cells do not adhere to the plastic and are removed when changing media. The remaining plastic adherent cells are termed MSCs, an abbreviation for both mesenchymal stem cells and mesenchymal stromal cells: Mesenchymal stromal cells, because they can contribute to the structural matrix of bone marrow and support hematopoesis; mesenchymal stem cells, because they have the ability to differentiate under ex vivo conditions into different mesenchymal-derived cells (Pittenger et al. 1999). MSCs may be isolated from bone marrow, skeletal muscle,

adipose tissue, synovial membranes and other connective tissue of human adults, as well as cord blood and placenta. Mesenchymal stem cells have been shown to inhibit inflammatory responses of innate and adaptive immune cells as well as have reparative effects on inflamed tissues.

Successful preclinical studies using MSCs in models of autoimmunity, inflammation and tissue damage have paved the way for clinical trials. In one trial, expanded autologous bone marrow mesenchymal stem cells (BM-MSC) were applied intravenously and recorded an improvement in CDAI in four out of six patients (Duijvestein et al. 2010). In a phase II trial with expanded allogeneic bone marrow derived adult MSCs that were administered in two doses in patients with moderate–severe refractory Crohn's disease (CDAI >220), a clinical response defined as a reduction in CDAI of at least 100 points in three out of nine patients was observed (Onken et al. 2006). In a 12-month study with eight patients, seven adverse events were reported (Onken et al. 2008). No tumors or formations of ectopic tissue were found. In another completed phase I trial of intravenous autologous bone marrow derived MSCs that included nine patients confirmed that the treatment is feasible and safe, but without apparent benefit for patients with severe refractory luminal disease (Duijvestein et al. 2010). At the moment, four clinical studies are registered at clinicaltrials.com to further investigate the safety and treatment outcome of intravenous human mesenchymal stem cells (Prochymal, remestemcel-L) as a therapy for Crohn's disease (NCT01510431, NCT01233960, NCT00294112, NCT00543374, NCT00482092).

Taken together, there is a rationale for testing MSCs in human IBD, but initial phase I and II studies have produced mixed results. Methodological concerns are the small numbers of patients, the variation in cell products and the lack of published controlled studies.

Regarding fistulizing disease, there were encouraging results from an initial phase I clinical trial using locally administered adipose-derived stem cells (ACS) to treat complex perianal fistula (Garcia-Olmo et al. 2005). These results have been confirmed in a phase II multicenter randomized trial including patients with complex perianal fistulas (cryptoglandular origin n = 35, associated with Crohn's disease n = 14) observing fistula closure in 17 out of 24 patients who received ASCs in addition to fibrin glue, compared to 4 out of 25 who received fibrin glue alone (Garcia-Olmo et al. 2009). Several studies are currently recruiting or completed assessing the use of bone marrow mesenchymal or adipose derived stem cells in fistulizing Crohn's disease (NCT01372969, NCT01011244, NCT01144962, NCT0992485, NCT01378390, NCT00999115, NCT01157650, NCT01314079).

In summary, it is important to define the source and type of MSC (autologous or allogenic) in order to standardize cell expansion conditions and to adopt uniformal study protocols. Also, significant issues remain regarding the design and interpretation, such as patient selection, disease stage, disease activity, MSC source (bone marrow, adipose tissue, placenta) and long-term safety. Although the perspective of immune reeducation and regulation seems fascinating, it is unrealistic to believe that cell-based therapies can eradicate immune disease, because most processes have a genetic predisposition that remain unaltered by an autologous transplant.

Especially for IBD, where the primary defect is probably a stem-cell differentiation problem in the intestine, new therapeutic strategies should be based on stimulation of the protective innate immune system.

39.3.2.3 Intestinal Stem Cells

Intestinal stem cells maintain the rapidly self-renewing intestinal tract tissue. Concerning the localisation of intestinal stem cells in the gut, there are two major models still under debate. The first is called the "+4 position model", which assumes that the stem cell is located above the Paneth cells at position +4 related to the crypt base (Haegebarth and Clevers 2009), the second is called the "stem cell zone model". This model proposes, that the crypt base columnar cells represent the intestinal stem cell (Haegebarth and Clevers 2009).

Intestinal stem cells differentiate into four epithelial cell types, namely absorptive columnar cells, goblet cells, neuroendocrine cells and Paneth cells. LGR5 (Leucine-rich-repeat-containing G-protein-coupled receptor 5) is a marker for intestinal stem cells since it could be shown that LGR5-positive cells are pluripotent, self-renewing and differentiate into all four epithelial cell types (Barker et al. 2007).

Paneth cells are located at the crypt of the cell and secrete defensins and other antimicrobial peptides to keep the crypt sterile. For ileal Crohn's disease it could be shown, that a defective differentiation from the intestinal stem cell toward the Paneth cell, because of a diminished expression of the Wnt signaling transcription factor TCF4 and the WNT coreceptor LRP6 (Koslowski et al. 2009, 2012), resulting in a defensin deficiency. This in turn leads to a dysfunctional mucosal barrier and an invasion of luminal microbes resulting in an inflamed mucosa. For ulcerative colitis, a defective differentiation from intestinal stem cell to goblet cell, mediated by the transcription factors Hath1 and KLF4, might lead to a goblet cell depletion and impaired mucin induction (Gersemann et al. 2009). This causes a defective mucus barrier and again invasion of luminal microbes triggering inflammation. Despite these exciting new perspectives, interventions focusing intestinal stem cells are difficult. One approach might be the genetic repair approach, supplying the defective genes to local crypt cells using a variety of gene transfer techniques. In a way, the alternatives of hematopoietic or mesenchymal versus intestinal stem cell therapies replays the current paradigm shift from adaptive to innate immunity centered on a barrier disease.

39.4 Conclusions and Future Perspectives on Regenerative Therapies

Medical therapies aiming at beta-cell neogenesis like gastrin, EGF, and INGAP (islet neogenesis associated protein) are in early stages of clinical development, with insufficient data available to date. Similarly, treatment of short bowel syndrome

with growth hormone with or without glutamine is ill-defined with regards to the optimal treatment regimen and safety. Intestinal growth factors are being tested for inflammatory bowel disease, and may be possibly of value as an adjunct therapy in the future. The GLP-2 analogon teduglutide, on the other hand, demonstrated promising results in the treatment of SBS and has been granted orphan status for this indication. Regarding cell-based regenerative approaches for T1D, pluripotent stem cells are still in preclinical development, and the first studies involving multipotent stem cells are ongoing. In inflammatory bowel disease, trials using hematopoietic or mesenchymal stem cells have reported limited efficacy. New promising approaches involve intestinal stem cells, which seem to account better for new clues to the pathogenesis of IBD as a barrier defect.

References

Abramowicz D, Schandene L, Goldman M, Crusiaux A, Vereerstraeten P, De Pauw L, Wybran J, Kinnaert P, Dupont E, Toussaint C (1989) Release of tumor necrosis factor, interleukin-2, and gamma-interferon in serum after injection of OKT3 monoclonal antibody in kidney transplant recipients. Transplantation 47:606–608

Agardh CD, Cilio CM, Lethagen A, Lynch K, Leslie RD, Palmer M, Harris RA, Robertson JA, Lernmark A (2005) Clinical evidence for the safety of GAD65 immunomodulation in adult-onset autoimmune diabetes. J Diabetes Complications 19:238–246

Agardh CD, Lynch KF, Palmer M, Link K, Lernmark A (2009) GAD65 vaccination: 5 years of follow-up in a randomised dose-escalating study in adult-onset autoimmune diabetes. Diabetologia 52:1363–1368

Anderson MS, Bluestone JA (2005) The NOD mouse: a model of immune dysregulation. Annu Rev Immunol 23:447–485

Ardizzone S, Bianchi PG (2002) Inflammatory bowel disease: new insights into pathogenesis and treatment. J Intern Med 252:475–496

Atkinson MA, Leiter EH (1999) The NOD mouse model of type 1 diabetes: as good as it gets? Nat Med 5:601–604

Atkinson MA, Maclaren NK (1994) The pathogenesis of insulin-dependent diabetes mellitus. N Engl J Med 331:1428–1436

Atkinson MA, Maclaren NK, Luchetta R (1990) Insulitis and diabetes in NOD mice reduced by prophylactic insulin therapy. Diabetes 39:933–937

Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449:1003–1007

Battaglia M, Stabilini A, Roncarolo MG (2005) Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. Blood 105:4743–4748

Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF, Chatenoud L (2003) TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. Nat Med 9:1202–1208

Birk OS, Elias D, Weiss AS, Rosen A, van der Zee R, Walker MD, Cohen IR (1996) NOD mouse diabetes: the ubiquitous mouse hsp60 is a beta-cell target antigen of autoimmune T cells. J Autoimmun 9:159

Bluestone JA, Herold K, Eisenbarth G (2010) Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature 464:1293–1300

Bockova J, Elias D, Cohen IR (1997) Treatment of NOD diabetes with a novel peptide of the hsp60 molecule induces Th2-type antibodies. J Autoimmun 10:323–329

Bonner-Weir S (1994) Regulation of pancreatic beta-cell mass in vivo. Recent Prog Horm Res 49:91–104

Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE (1993) A second pathway for regeneration of adult exocrine and endocrine pancreas: a possible recapitulation of embryonic development. Diabetes 42:1715–1720

Bonner-Weir S, Li WC, Ouziel-Yahalom L, Guo L, Weir GC, Sharma A (2010) Beta-cell growth and regeneration: replication is only part of the story. Diabetes 59:2340–2348

Bougneres PF, Landais P, Boisson C, Carel JC, Frament N, Boitard C, Chaussain JL, Bach JF (1990) Limited duration of remission of insulin dependency in children with recent overt type I diabetes treated with low-dose cyclosporin. Diabetes 39:1264–1272

Bouwens L, Pipeleers DG (1998) Extra-insular beta cells associated with ductules are frequent in adult human pancreas. Diabetologia 41:629–633

Bresson D, von Herrath M (2007) Moving towards efficient therapies in type 1 diabetes: to combine or not to combine? Autoimmun Rev 6:315–322

Bresson D, Togher L, Rodrigo E, Chen Y, Bluestone JA, Herold KC, von Herrath M (2006) Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs. J Clin Invest 116:1371–1381

Brignier AC, Gewirtz AM (2010) Embryonic and adult stem cell therapy. J Allergy Clin Immunol 125:S336–S344

Buchman AL, Katz S, Fang JC, Bernstein CN, Abou-Assi SG (2010) Teduglutide, a novel mucosally active analog of glucagon-like peptide-2 (GLP-2) for the treatment of moderate to severe Crohn's disease. Inflamm Bowel Dis 16:962–973

Burt RK, Craig RM, Milanetti F, Quigley K, Gozdziak P, Bucha J, Testori A, Halverson A, Verda L, de Villiers WJ, Jovanovic B, Oyama Y (2010) Autologous nonmyeloablative hematopoietic stem cell transplantation in patients with severe anti-TNF refractory Crohn disease: long-term follow-up. Blood 116:6123–6132

Buse JB, Henry RR, Han J, Kim DD, Fineman MS, Baron AD (2004) Effects of exenatide (exendin-4) on glycemic control over 30 weeks in sulfonylurea-treated patients with type 2 diabetes. Diabetes Care 27:2628–2635

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC (2003a) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 52:102–110

Butler AE, Janson J, Soeller WC, Butler PC (2003b) Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid. Diabetes 52:2304–2314

Byrne TA, Morrissey TB, Nattakom TV, Ziegler TR, Wilmore DW (1995a) Growth hormone, glutamine, and a modified diet enhance nutrient absorption in patients with severe short bowel syndrome. JPEN J Parenter Enteral Nutr 19:296–302

Byrne TA, Persinger RL, Young LS, Ziegler TR, Wilmore DW (1995b) A new treatment for patients with short-bowel syndrome. Growth hormone, glutamine, and a modified diet. Ann Surg 222:243–254

Cardona K, Milas Z, Strobert E, Cano J, Jiang W, Safley SA, Gangappa S, Hering BJ, Weber CJ, Pearson TC, Larsen CP (2007) Engraftment of adult porcine islet xenografts in diabetic nonhuman primates through targeting of costimulation pathways. Am J Transplant 7:2260–2268

Carteron NL, Schimenti CL, Wofsy D (1989) Treatment of murine lupus with F(ab')2 fragments of monoclonal antibody to L3T4. Suppression of autoimmunity does not depend on T helper cell depletion. J Immunol 142:1470–1475

Cassinotti A, Annaloro C, Ardizzone S, Onida F, Della VA, Clerici M, Usardi P, Greco S, Maconi G, Porro GB, Deliliers GL (2008) Autologous haematopoietic stem cell transplantation without CD34+ cell selection in refractory Crohn's disease. Gut 57:211–217

Chatenoud L, Baudrihaye MF, Kreis H, Goldstein G, Schindler J, Bach JF (1982) Human in vivo antigenic modulation induced by the anti-T cell OKT3 monoclonal antibody. Eur J Immunol 12:979–982

Chatenoud L, Thervet E, Primo J, Bach JF (1994) Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci USA 91:123–127

Chatenoud L, Primo J, Bach JF (1997) CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. J Immunol 158:2947–2954
Chen C, Lee WH, Yun P, Snow P, Liu CP (2003) Induction of autoantigen-specific Th2 and Tr1 regulatory T cells and modulation of autoimmune diabetes. J Immunol 171:733–744
Chung CH, Hao E, Piran R, Keinan E, Levine F (2010) Pancreatic beta-cell neogenesis by direct conversion from mature alpha-cells. Stem Cells 28:1630–1638
Cosimi AB, Burton RC, Colvin RB, Goldstein G, Delmonico FL, LaQuaglia MP, Tolkoff-Rubin N, Rubin RH, Herrin JT, Russell PS (1981) Treatment of acute renal allograft rejection with OKT3 monoclonal antibody. Transplantation 32:535–539
Couri CE, Voltarelli JC (2009) Stem cell therapy for type 1 diabetes mellitus: a review of recent clinical trials. Diabetol Metab Syndr 1:19
Couri CE, Oliveira MC, Stracieri AB, Moraes DA, Pieroni F, Barros GM, Madeira MI, Malmegrim KC, Foss-Freitas MC, Simoes BP, Martinez EZ, Foss MC, Burt RK, Voltarelli JC (2009) C-peptide levels and insulin independence following autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 301:1573–1579
Couzin-Frankel J (2010) Immunology. Replacing an immune system gone haywire. Science 327:772–774
Craig RM, Traynor A, Oyama Y, Burt RK (2003) Hematopoietic stem cell transplantation for severe Crohn's disease. Bone Marrow Transplant 32(Suppl 1):S57–S59
Cucca F, Muntoni F, Lampis R, Frau F, Argiolas L, Silvetti M, Angius E, Cao A, De Virgiliis S, Congia M (1993) Combinations of specific DRB1, DQA1, DQB1 haplotypes are associated with insulin-dependent diabetes mellitus in Sardinia. Hum Immunol 37:85–94
Dahly EM, Miller ME, Lund PK, Ney DM (2004) Postreceptor resistance to exogenous growth hormone exists in the jejunal mucosa of parenterally fed rats. J Nutr 134:530–537
Daneman D (2006) Type 1 diabetes. Lancet 367:847–858
Daniel D, Wegmann DR (1996) Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9–23). Proc Natl Acad Sci USA 93:956–960
Denson LA, Kim MO, Bezold R, Carey R, Osuntokun B, Nylund C, Willson T, Bonkowski E, Li D, Ballard E, Collins M, Moyer MS, Klein DJ (2010) A randomized controlled trial of growth hormone in active pediatric Crohn disease. J Pediatr Gastroenterol Nutr 51:130–139
Dieckgraefe BK, Korzenik JR (2002) Treatment of active Crohn's disease with recombinant human granulocyte-macrophage colony-stimulating factor. Lancet 360:1478–1480
Ditschkowski M, Einsele H, Schwerdtfeger R, Bunjes D, Trenschel R, Beelen DW, Elmaagacli AH (2003) Improvement of inflammatory bowel disease after allogeneic stem-cell transplantation. Transplantation 75:1745–1747
Dor Y, Brown J, Martinez OI, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429:41–46
Drakos PE, Nagler A, Or R (1993) Case of Crohn's disease in bone marrow transplantation. Am J Hematol 43:157–158
Drucker DJ (2003) Glucagon-like peptides: regulators of cell proliferation, differentiation, and apoptosis. Mol Endocrinol 17:161–171
Drucker DJ, Erlich P, Asa SL, Brubaker PL (1996) Induction of intestinal epithelial proliferation by glucagon-like peptide 2. Proc Natl Acad Sci USA 93:7911–7916
Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH, Verhaar AP, Fibbe WE, van den Brink GR, Hommes DW (2010) Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut 59:1662–1669
Dungan KM, Buse JB, Ratner RE (2009) Effects of therapy in type 1 and type 2 diabetes mellitus with a peptide derived from islet neogenesis associated protein (INGAP). Diabetes Metab Res Rev. 25:558–565
Edinger M, Hoffmann P (2011) Regulatory T cells in stem cell transplantation: strategies and first clinical experiences. Curr Opin Immunol 23:679–684
Elias D, Cohen IR (1994) Peptide therapy for diabetes in NOD mice. Lancet 343:704–706

Elias D, Meilin A, Ablamunits V, Birk OS, Carmi P, Konen-Waisman S, Cohen IR (1997) Hsp60 peptide therapy of NOD mouse diabetes induces a Th2 cytokine burst and downregulates autoimmunity to various beta-cell antigens. Diabetes 46:758–764

Elinav E, Adam N, Waks T, Eshhar Z (2009) Amelioration of colitis by genetically engineered murine regulatory T cells redirected by antigen-specific chimeric receptor. Gastroenterology 136:1721–1731

Ellegard L, Bosaeus I, Nordgren S, Bengtsson BA (1997) Low-dose recombinant human growth hormone increases body weight and lean body mass in patients with short bowel syndrome. Ann Surg 225:88–96

Faria AM, Weiner HL (2006a) Oral tolerance and TGF-beta-producing cells. Inflamm Allergy Drug Targets 5:179–190

Faria AM, Weiner HL (2006b) Oral tolerance: therapeutic implications for autoimmune diseases. Clin Dev Immunol 13:143–157

Farilla L, Bulotta A, Hirshberg B, Li Calzi S, Khoury N, Noushmehr H, Bertolotto C, Di Mario U, Harlan DM, Perfetti R (2003) Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. Endocrinology 144:5149–5158

Fenalti G, Rowley MJ (2008) GAD65 as a prototypic autoantigen. J Autoimmun 31:228–232

Feutren G, Papoz L, Assan R, Vialettes B, Karsenty G, Vexiau P, Du Rostu H, Rodier M, Sirmai J, Lallemand A et al (1986) Cyclosporin increases the rate and length of remissions in insulin-dependent diabetes of recent onset. Results of a multicentre double-blind trial. Lancet 2:119–124

Fiocchi C (1997) Intestinal inflammation: a complex interplay of immune and nonimmune cell interactions. Am J Physiol 273:G769–G775

Fiorina P, Voltarelli J, Zavazava N (2011) Immunological applications of stem cells in type 1 diabetes. Endocr Rev 32:725–754

Flint JM (1912) The effect of extensive resection of small intestine. Bull Johns Hopkins Hosp 23:127–144

Flomenberg N, Devine SM, Dipersio JF, Liesveld JL, McCarty JM, Rowley SD, Vesole DH, Badel K, Calandra G (2005) The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. Blood 106:1867–1874

Fraenkel M, Ketzinel-Gilad M, Ariav Y, Pappo O, Karaca M, Castel J, Berthault MF, Magnan C, Cerasi E, Kaiser N, Leibowitz G (2008) mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes. Diabetes 57:945–957

Friel R, van der Sar S, Mee PJ (2005) Embryonic stem cells: understanding their history, cell biology and signalling. Adv Drug Deliv Rev 57:1894–1903

Friend PJ, Hale G, Chatenoud L, Rebello P, Bradley J, Thiru S, Phillips JM, Waldmann H (1999) Phase I study of an engineered aglycosylated humanized CD3 antibody in renal transplant rejection. Transplantation 68:1632–1637

Garcia-Olmo D, Garcia-Arranz M, Herreros D, Pascual I, Peiro C, Rodriguez-Montes JA (2005) A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. Dis Colon Rectum 48:1416–1423

Garcia-Olmo D, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, De La Quintana P, Garcia-Arranz M, Pascual M (2009) Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. Dis Colon Rectum 52:79–86

Garkavenko O, Dieckhoff B, Wynyard S, Denner J, Elliott RB, Tan PL, Croxson MC (2008) Absence of transmission of potentially xenotic viruses in a prospective pig to primate islet xenotransplantation study. J Med Virol 80:2046–2052

Gersemann M, Becker S, Kubler I, Koslowski M, Wang G, Herrlinger KR, Griger J, Fritz P, Fellermann K, Schwab M, Wehkamp J, Stange EF (2009) Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. Differentiation 77:84–94

Gersemann M, Wehkamp J, Stange EF (2012) Innate immune dysfunction in inflammatory bowel disease. J Intern Med 271:421–428

Gianani R, Eisenbarth GS (2005) The stages of type 1A diabetes: 2005. Immunol Rev 204:232–249

Gillespie KM (2006) Type 1 diabetes: pathogenesis and prevention. CMAJ 175:165–170
Gilroy R, Allard JP, Jeppesen PB, Seidner P, Pertkiewicz M, Howard L, O'Keefe SJ, Messing B, Jeejeebhoy K (2008) Treatment out to 1 year with a GLP-2 analog, teduglutide, safely reduces parenteral nutrition (PN) needs in PN-dependent short bowel syndrome (SBS) patients. Am J Gastroenterol 103:S105–S106
Ginsberg-Fellner F, Witt ME, Fedun B, Taub F, Dobersen MJ, McEvoy RC, Cooper LZ, Notkins AL, Rubinstein P (1985) Diabetes mellitus and autoimmunity in patients with the congenital rubella syndrome. Rev Infect Dis 7(Suppl 1):S170–S176
Glocker EO, Kotlarz D, Boztug K, Gertz EM, Schaffer AA, Noyan F, Perro M, Diestelhorst J, Allroth A, Murugan D, Hatscher N, Pfeifer D, Sykora KW, Sauer M, Kreipe H, Lacher M, Nustede R, Woellner C, Baumann U, Salzer U, Koletzko S, Shah N, Segal AW, Sauerbrey A, Buderus S, Snapper SB, Grimbacher B, Klein C (2009) Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N Engl J Med 361:2033–2045
Gouttebel MC, Astre C, Briand D, Saint-Aubert B, Girardot PM, Joyeux H (1992) Influence of N-acetylglutamine or glutamine infusion on plasma amino acid concentrations during the early phase of small-bowel adaptation in the dog. JPEN J Parenter Enteral Nutr 16:117–121
Gratwohl A, Brand R, Frassoni F, Rocha V, Niederwieser D, Reusser P, Einsele H, Cordonnier C (2005) Cause of death after allogeneic haematopoietic stem cell transplantation (HSCT) in early leukaemias: an EBMT analysis of lethal infectious complications and changes over calendar time. Bone Marrow Transplant 36:757–769
Gruessner AC, Sutherland DE (2008) Pancreas transplant outcomes for United States (US) cases as reported to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR). Clin Transpl 2008:45–56
Gu Y, Wu ZH, Xie JX, Jin DY, Zhuo HC (2001) Effects of growth hormone (rhGH) and glutamine supplemented parenteral nutrition on intestinal adaptation in short bowel rats. Clin Nutr 20:159–166
Guan X, Karpen HE, Stephens J, Bukowski JT, Niu S, Zhang G, Stoll B, Finegold MJ, Holst JJ, Hadsell D, Nichols BL, Burrin DG (2006) GLP-2 receptor localizes to enteric neurons and endocrine cells expressing vasoactive peptides and mediates increased blood flow. Gastroenterology 130:150–164
Guleria I, Gubbels Bupp M, Dada S, Fife B, Tang Q, Ansari MJ, Trikudanathan S, Vadivel N, Fiorina P, Yagita H, Azuma M, Atkinson M, Bluestone JA, Sayegh MH (2007) Mechanisms of PDL1-mediated regulation of autoimmune diabetes. Clin Immunol 125:16–25
Guo Z, Wu T, Kirchhof N, Mital D, Williams JW, Azuma M, Sutherland DE, Hering BJ (2001) Immunotherapy with nondepleting anti-CD4 monoclonal antibodies but not CD28 antagonists protects islet graft in spontaneously diabetic nod mice from autoimmune destruction and allogeneic and xenogeneic graft rejection. Transplantation 71:1656–1665
Gutstein NL, Seaman WE, Scott JH, Wofsy D (1986) Induction of immune tolerance by administration of monoclonal antibody to L3T4. J Immunol 137:1127–1132
Haegebarth A, Clevers H (2009) Wnt signaling, lgr5, and stem cells in the intestine and skin. Am J Pathol 174:715–721
Harrison LC, Dempsey-Collier M, Kramer DR, Takahashi K (1996) Aerosol insulin induces regulatory CD8 gamma delta T cells that prevent murine insulin-dependent diabetes. J Exp Med 184:2167–2174
Harrison LC, Honeyman MC, Steele CE, Stone NL, Sarugeri E, Bonifacio E, Couper JJ, Colman PG (2004) Pancreatic beta-cell function and immune responses to insulin after administration of intranasal insulin to humans at risk for type 1 diabetes. Diabetes Care 27:2348–2355
Herold KC, Bluestone JA (2011) Type 1 diabetes immunotherapy: is the glass half empty or half full? Sci Transl Med 3(95):95fs1
Hjorth M, Axelsson S, Ryden A, Faresjo M, Ludvigsson J, Casas R (2011) GAD-alum treatment induces GAD65-specific CD4+CD25highFOXP3+ cells in type 1 diabetic patients. Clin Immunol 138:117–126
Horvath L, Cervenak L, Oroszlan M, Prohaszka Z, Uray K, Hudecz F, Baranyi E, Madacsy L, Singh M, Romics L, Fust G, Panczel P (2002) Antibodies against different epitopes of heat-shock protein 60 in children with type 1 diabetes mellitus. Immunol Lett 80:155–162

Hu CY, Rodriguez-Pinto D, Du W, Ahuja A, Henegariu O, Wong FS, Shlomchik MJ, Wen L (2007) Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. J Clin Invest 117:3857–3867

Iakoubov R, Lauffer LM, Trivedi S, Kim YI, Brubaker PL (2009) Carcinogenic effects of exogenous and endogenous glucagon-like peptide-2 in azoxymethane-treated mice. Endocrinology 150:4033–4043

Ilonen J, Sjoroos M, Knip M, Veijola R, Simell O, Akerblom HK, Paschou P, Bozas E, Havarani B, Malamitsi-Puchner A, Thymelli J, Vazeou A, Bartsocas CS (2002) Estimation of genetic risk for type 1 diabetes. Am J Med Genet 115:30–36

Inoue Y, Copeland EM, Souba WW (1994) Growth hormone enhances amino acid uptake by the human small intestine. Ann Surg 219:715–722

Jacobs LR (1983) Effects of dietary fiber on mucosal growth and cell proliferation in the small intestine of the rat: a comparison of oat bran, pectin, and guar with total fiber deprivation. Am J Clin Nutr 37:954–960

Jaeckel E, Mpofu N, Saal N, Manns MP (2008) Role of regulatory T cells for the treatment of type 1 diabetes mellitus. Horm Metab Res 40:126–136

Jena B, Dotti G, Cooper LJ (2010) Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. Blood 116:1035–1044

Jeppesen PB, Szkudlarek J, Hoy CE, Mortensen PB (2001) Effect of high-dose growth hormone and glutamine on body composition, urine creatinine excretion, fatty acid absorption, and essential fatty acids status in short bowel patients: a randomized, double-blind, crossover, placebo-controlled study. Scand J Gastroenterol 36:48–54

Jeppesen PB, Sanguinetti EL, Buchman A, Howard L, Scolapio JS, Ziegler TR, Gregory J, Tappenden KA, Holst J, Mortensen PB (2005) Teduglutide (ALX-0600), a dipeptidyl peptidase IV resistant glucagon-like peptide 2 analogue, improves intestinal function in short bowel syndrome patients. Gut 54:1224–1231

Jeppesen PB, Gilroy R, Pertkiewicz M, Allard JP, Messing B, O'Keefe SJ (2011) Randomised placebo-controlled trial of teduglutide in reducing parenteral nutrition and/or intravenous fluid requirements in patients with short bowel syndrome. Gut 60:902–914

Kapoor S, Wilson AG, Sharrack B, Lobo A, Akil M, Sun L, Dalley CD, Snowden JA (2007) Haemopoietic stem cell transplantation–an evolving treatment for severe autoimmune and inflammatory diseases in rheumatology, neurology and gastroenterology. Hematology 12:179–191

Katz JD, Wang B, Haskins K, Benoist C, Mathis D (1993) Following a diabetogenic T cell from genesis through pathogenesis. Cell 74:1089–1100

Kazkaz H, Isenberg D (2004) Anti B cell therapy (rituximab) in the treatment of autoimmune diseases. Curr Opin Pharmacol 4:398–402

Kishimoto H, Sprent J (2001) A defect in central tolerance in NOD mice. Nat Immunol 2:1025–1031

Kobayashi T, Arefanian H, Harb G, Tredget EB, Rajotte RV, Korbutt GS, Rayat GR (2008) Prolonged survival of microencapsulated neonatal porcine islet xenografts in immune-competent mice without antirejection therapy. Cell Transplant 17:1243

Kolterman OG, Buse JB, Fineman MS, Gaines E, Heintz S, Bicsak TA, Taylor K, Kim D, Aisporna M, Wang Y, Baron AD (2003) Synthetic exendin-4 (exenatide) significantly reduces postprandial and fasting plasma glucose in subjects with type 2 diabetes. J Clin Endocrinol Metab 88:3082–3089

Korzenik JR, Dieckgraefe BK, Valentine JF, Hausman DF, Gilbert MJ (2005) Sargramostim for active Crohn's disease. N Engl J Med 352:2193–2201

Koslowski MJ, Kubler I, Chamaillard M, Schaeffeler E, Reinisch W, Wang G, Beisner J, Teml A, Peyrin-Biroulet L, Winter S, Herrlinger KR, Rutgeerts P, Vermeire S, Cooney R, Fellermann K, Jewell D, Bevins CL, Schwab M, Stange EF, Wehkamp J (2009) Genetic variants of Wnt transcription factor TCF-4 (TCF7L2) putative promoter region are associated with small intestinal Crohn's disease. PLoS One 4:e4496

Koslowski MJ, Teltschik Z, Beisner J, Schaeffeler E, Wang G, Kübler I, Gersemann M, Cooney R, Jewell D, Reinisch W, Vermeire S, Rutgeerts P, Schwab M, Stange EF, Wehkamp J (2012)

Association of a functional variant in the Wnt co-receptor LRP6 with early onset ileal Crohn's disease. PLoS Genet 8:e1002523
Kreisel W, Potthoff K, Bertz H, Schmitt-Graeff A, Ruf G, Rasenack J, Finke J (2003) Complete remission of Crohn's disease after high-dose cyclophosphamide and autologous stem cell transplantation. Bone Marrow Transplant 32:337–340
Kremer JM, Genant HK, Moreland LW, Russell AS, Emery P, Abud-Mendoza C, Szechinski J, Li T, Teng J, Becker JC, Westhovens R (2008) Results of a two-year followup study of patients with rheumatoid arthritis who received a combination of abatacept and methotrexate. Arthritis Rheum 58:953–963
Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, Young H, Richardson M, Smart NG, Cunningham J, Agulnick AD, D'Amour KA, Carpenter MK, Baetge EE (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 26:443–452
Kupila A, Sipila J, Keskinen P, Simell T, Knip M, Pulkki K, Simell O (2003) Intranasally administered insulin intended for prevention of type 1 diabetes–a safety study in healthy adults. Diabetes Metab Res Rev 19:415–420
Lazar L, Ofan R, Weintrob N, Avron A, Tamir M, Elias D, Phillip M, Josefsberg Z (2007) Heat-shock protein peptide DiaPep277 treatment in children with newly diagnosed type 1 diabetes: a randomised, double-blind phase II study. Diabetes Metab Res Rev 23:286–291
Lee RH, Seo MJ, Reger RL, Spees JL, Pulin AA, Olson SD, Prockop DJ (2006) Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. Proc Natl Acad Sci USA 103:17438–17443
Li L, El-Kholy W, Rhodes CJ, Brubaker PL (2005) Glucagon-like peptide-1 protects beta cells from cytokine-induced apoptosis and necrosis: role of protein kinase B. Diabetologia 48:1339–1349
Li Y, Zhang R, Qiao H, Zhang H, Wang Y, Yuan H, Liu Q, Liu D, Chen L, Pei X (2007) Generation of insulin-producing cells from PDX-1 gene-modified human mesenchymal stem cells. J Cell Physiol 211:36–44
Lipsett M, Hanley S, Castellarin M, Austin E, Suarez-Pinzon WL, Rabinovitch A, Rosenberg L (2007) The role of islet neogenesis-associated protein (INGAP) in islet neogenesis. Cell Biochem Biophys 48:127–137
Looney RJ (2005) B cells as a therapeutic target in autoimmune diseases other than rheumatoid arthritis. Rheumatology (Oxford) 44(Suppl 2):ii13–ii17
Lopez-Cubero SO, Sullivan KM, McDonald GB (1998) Course of Crohn's disease after allogeneic marrow transplantation. Gastroenterology 114:433–440
Ludvigsson J, Faresjo M, Hjorth M, Axelsson S, Cheramy M, Pihl M, Vaarala O, Forsander G, Ivarsson S, Johansson C, Lindh A, Nilsson NO, Aman J, Ortqvist E, Zerhouni P, Casas R (2008) GAD treatment and insulin secretion in recent-onset type 1 diabetes. N Engl J Med 359:1909–1920
Lund PK (1998) Molecular basis of intestinal adaptation: the role of the insulin-like growth factor system. Ann N Y Acad Sci 859:18–36
Ma SW, Zhao DL, Yin ZQ, Mukherjee R, Singh B, Qin HY, Stiller CR, Jevnikar AM (1997) Transgenic plants expressing autoantigens fed to mice to induce oral immune tolerance. Nat Med 3:793–796
Madec AM, Mallone R, Afonso G, Abou Mrad E, Mesnier A, Eljaafari A, Thivolet C (2009) Mesenchymal stem cells protect NOD mice from diabetes by inducing regulatory T cells. Diabetologia 52:1391–1399
Maehr R (2011) iPS cells in type 1 diabetes research and treatment. Clin Pharmacol Ther 89:750–753
Masteller EL, Warner MR, Tang Q, Tarbell KV, McDevitt H, Bluestone JA (2005) Expansion of functional endogenous antigen-specific CD4+CD25+ regulatory T cells from nonobese diabetic mice. J Immunol 175:3053–3059
Matveyenko AV, Georgia S, Bhushan A, Butler PC (2010) Inconsistent formation and nonfunction of insulin-positive cells from pancreatic endoderm derived from human embryonic stem cells in athymic nude rats. Am J Physiol Endocrinol Metab 299:E713–E720

McCall MD, Toso C, Baetge EE, Shapiro AM (2009) Are stem cells a cure for diabetes? Clin Sci (Lond) 118:87–97

Monti P, Scirpoli M, Maffi P, Piemonti L, Secchi A, Bonifacio E, Roncarolo MG, Battaglia M (2008) Rapamycin monotherapy in patients with type 1 diabetes modifies CD4 + CD25 + FOXP3+ regulatory T-cells. Diabetes 57:2341–2347

Morgan RA, Dudley ME, Rosenberg SA (2010) Adoptive cell therapy: genetic modification to redirect effector cell specificity. Cancer J 16:336–341

Muir A, Peck A, Clare-Salzler M, Song YH, Cornelius J, Luchetta R, Krischer J, Maclaren N (1995) Insulin immunization of nonobese diabetic mice induces a protective insulitis characterized by diminished intraislet interferon-gamma transcription. J Clin Invest 95:628–634

Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, Yu L, Wegmann DR, Hutton JC, Elliott JF, Eisenbarth GS (2005) Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. Nature 435:220–223

Nejentsev S, Sjoroos M, Soukka T, Knip M, Simell O, Lovgren T, Ilonen J (1999) Population-based genetic screening for the estimation of Type 1 diabetes mellitus risk in Finland: selective genotyping of markers in the HLA-DQB1, HLA-DQA1 and HLA-DRB1 loci. Diabet Med 16:985–992

Nightingale JM, Lennard-Jones JE (1993) The short bowel syndrome: what's new and old? Dig Dis 11:12–31

Noble JA, Valdes AM, Cook M, Klitz W, Thomson G, Erlich HA (1996) The role of HLA class II genes in insulin-dependent diabetes mellitus: molecular analysis of 180 Caucasian, multiplex families. Am J Hum Genet 59:1134–1148

Ogawa N, List JF, Habener JF, Maki T (2004) Cure of overt diabetes in NOD mice by transient treatment with anti-lymphocyte serum and exendin-4. Diabetes 53:1700–1705

Onken J, Gallup D, Hanson J (2006). Successful outpatient treatment of refractory Crohn's disease using adult mesenchymal stem cells. ACG 2006 Final Program Book p. 121

Onken J, Jaffe T, Custer L (2008) Long-term safety of prochymal adult mesenchymal stem cells in Crohn's disease. Gastroenterology 134:A–66

Orskov C, Hartmann B, Poulsen SS, Thulesen J, Hare KJ, Holst JJ (2005) GLP-2 stimulates colonic growth via KGF, released by subepithelial myofibroblasts with GLP-2 receptors. Regul Pept 124:105–112

Oyama Y, Craig RM, Traynor AE, Quigley K, Statkute L, Halverson A, Brush M, Verda L, Kowalska B, Krosnjar N, Kletzel M, Whitington PF, Burt RK (2005) Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. Gastroenterology 128:552–563

Parving HH, Tarnow L, Nielsen FS, Rossing P, Mandrup-Poulsen T, Osterby R, Nerup J (1999) Cyclosporine nephrotoxicity in type 1 diabetic patients. A 7-year follow-up study. Diabetes Care 22:478–833

Patterson CC, Dahlquist GG, Gyurus E, Green A, Soltesz G (2009) Incidence trends for childhood type 1 diabetes in Europe during 1989–2003 and predicted new cases 2005–20: a multicentre prospective registration study. Lancet 373:2027–2033

Peltola H, Davidkin I, Paunio M, Valle M, Leinikki P, Heinonen OP (2000) Mumps and rubella eliminated from Finland. JAMA 284:2643–2647

Pironi L, Forbes A, Joly F, Colomb V, Lyszkowska M, Van GA, Baxter J, Thul P, Hebuterne X, Gambarara M, Gottrand F, Moreno Villares JM, Messing B, Goulet O, Staun M (2008) Survival of patients identified as candidates for intestinal transplantation: a 3-year prospective follow-up. Gastroenterology 135:61–71

Pironi L, Joly F, Forbes A, Colomb V, Lyszkowska M, Baxter J, Gabe S, Hebuterne X, Gambarara M, Gottrand F, Cuerda C, Thul P, Messing B, Goulet O, Staun M, Van GA (2011) Long-term follow-up of patients on home parenteral nutrition in Europe: implications for intestinal transplantation. Gut 60:17–25

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Playford RJ, Marchbank T, Goodlad RA, Chinery RA, Poulsom R, Hanby AM (1996) Transgenic mice that overexpress the human trefoil peptide pS2 have an increased resistance to intestinal damage. Proc Natl Acad Sci USA 93:2137–2142

Pozzilli P (2002) The DPT-1 trial: a negative result with lessons for future type 1 diabetes prevention. Diabetes Metab Res Rev 18:257–259

Pozzilli P, Pitocco D, Visalli N, Cavallo MG, Buzzetti R, Crino A, Spera S, Suraci C, Multari G, Cervoni M, Manca Bitti ML, Matteoli MC, Marietti G, Ferrazzoli F, Cassone Faldetta MR, Giordano C, Sbriglia M, Sarugeri E, Ghirlanda G (2000) No effect of oral insulin on residual beta-cell function in recent-onset type I diabetes (the IMDIAB VII) (IMDIAB Group). Diabetologia 43:1000–1004

Raz I, Elias D, Avron A, Tamir M, Metzger M, Cohen IR (2001) Beta-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial. Lancet 358:1749–1753

Raz I, Avron A, Tamir M, Metzger M, Symer L, Eldor R, Cohen IR, Elias D (2007) Treatment of new-onset type 1 diabetes with peptide DiaPep277 is safe and associated with preserved beta-cell function: extension of a randomized, double-blind, phase II trial. Diabetes Metab Res Rev 23:292–298

Rigby MR, Trexler AM, Pearson TC, Larsen CP (2008) CD28/CD154 blockade prevents autoimmune diabetes by inducing nondeletional tolerance after effector t-cell inhibition and regulatory T-cell expansion. Diabetes 57:2672–2683

Roep BO, Atkinson M, von Herrath M (2004) Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes. Nat Rev Immunol 4:989–997

Rooman I, Bouwens L (2004) Combined gastrin and epidermal growth factor treatment induces islet regeneration and restores normoglycaemia in C57Bl6/J mice treated with alloxan. Diabetologia 47:259–265

Rooman I, Lardon J, Bouwens L (2002) Gastrin stimulates beta-cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreas tissue. Diabetes 51:686–690

Rosenberg L, Lipsett M, Yoon JW, Prentki M, Wang R, Jun HS, Pittenger GL, Taylor-Fishwick D, Vinik AI (2004) A pentadecapeptide fragment of islet neogenesis-associated protein increases beta-cell mass and reverses diabetes in C57BL/6 J mice. Ann Surg 240:875–884

Rother KI, Spain LM, Wesley RA, Digon BJ III, Baron A, Chen K, Nelson P, Dosch HM, Palmer JP, Brooks-Worrell B, Ring M, Harlan DM (2009) Effects of exenatide alone and in combination with daclizumab on beta-cell function in long-standing type 1 diabetes. Diabetes Care 32:2251–2257

Rowland KJ, Brubaker PL (2011) The "cryptic" mechanism of action of glucagon-like peptide-2. Am J Physiol Gastrointest Liver Physiol 301:G1–G8

Salomon B, Bluestone JA (2001) Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu Rev Immunol 19:225–252

Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA (2000) B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 12:431–440

Sandborn WJ, Sands BE, Wolf DC, Valentine JF, Safdi M, Katz S, Isaacs KL, Wruble LD, Katz J, Present DH, Loftus EV Jr, Graeme-Cook F, Odenheimer DJ, Hanauer SB (2003) Repifermin (keratinocyte growth factor-2) for the treatment of active ulcerative colitis: a randomized, double-blind, placebo-controlled, dose-escalation trial. Aliment Pharmacol Ther 17:1355–1364

Scheppach W, Loges C, Bartram P, Christl SU, Richter F, Dusel G, Stehle P, Fuerst P, Kasper H (1994) Effect of free glutamine and alanyl-glutamine dipeptide on mucosal proliferation of the human ileum and colon. Gastroenterology 107:429–434

Scolapio JS, Camilleri M, Fleming CR, Oenning LV, Burton DD, Sebo TJ, Batts KP, Kelly DG (1997) Effect of growth hormone, glutamine, and diet on adaptation in short-bowel syndrome: a randomized, controlled study. Gastroenterology 113:1074–1081

Scott RB, Kirk D, MacNaughton WK, Meddings JB (1998) GLP-2 augments the adaptive response to massive intestinal resection in rat. Am J Physiol 275:G911–G921

Seguy D, Vahedi K, Kapel N, Souberbielle JC, Messing B (2003) Low-dose growth hormone in adult home parenteral nutrition-dependent short bowel syndrome patients: a positive study. Gastroenterology 124:293–302

Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, Borg C, Saas P, Tiberghien P, Rouas-Freiss N, Carosella ED, Deschaseaux F (2008) Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. Stem Cells 26:212–222

Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 343:230–238

Sherry NA, Chen W, Kushner JA, Glandt M, Tang Q, Tsai S, Santamaria P, Bluestone JA, Brillantes AM, Herold KC (2007) Exendin-4 improves reversal of diabetes in NOD mice treated with anti-CD3 monoclonal antibody by enhancing recovery of beta-cells. Endocrinology 148: 5136–5144

Silverstein J, Maclaren N, Riley W, Spillar R, Radjenovic D, Johnson S (1988) Immunosuppression with azathioprine and prednisone in recent-onset insulin-dependent diabetes mellitus. N Engl J Med 319:599–604

Sinha A, Nightingale J, West KP, Berlanga-Acosta J, Playford RJ (2003) Epidermal growth factor enemas with oral mesalamine for mild-to-moderate left-sided ulcerative colitis or proctitis. N Engl J Med 349:350–357

Slonim AE, Bulone L, Damore MB, Goldberg T, Wingertzahn MA, McKinley MJ (2000) A preliminary study of growth hormone therapy for Crohn's disease. N Engl J Med 342:1633–1637

Snarski E, Milczarczyk A, Torosian T, Paluszewska M, Urbanowska E, Krol M, Boguradzki P, Jedynasty K, Franek E, Wiktor-Jedrzejczak W (2011) Independence of exogenous insulin following immunoablation and stem cell reconstitution in newly diagnosed diabetes type I. Bone Marrow Transplant 46:562–566

Stefanovic S, Abboud N, Desilets S, Nury D, Cowan C, Puceat M (2009) Interplay of Oct4 with Sox2 and Sox17: a molecular switch from stem cell pluripotency to specifying a cardiac fate. J Cell Biol 186:665–673

Stiller CR, Dupre J, Gent M, Heinrichs D, Jenner MR, Keown PA, Laupacis A, Martell R, Rodger NW, Wolfe BM et al (1987) Effects of cyclosporine in recent-onset juvenile type 1 diabetes: impact of age and duration of disease. J Pediatr 111:1069–1072

Suarez-Pinzon WL, Yan Y, Power R, Brand SJ, Rabinovitch A (2005) Combination therapy with epidermal growth factor and gastrin increases beta-cell mass and reverses hyperglycemia in diabetic NOD mice. Diabetes 54:2596–2601

Suarez-Pinzon WL, Power RF, Yan Y, Wasserfall C, Atkinson M, Rabinovitch A (2008) Combination therapy with glucagon-like peptide-1 and gastrin restores normoglycemia in diabetic NOD mice. Diabetes 57:3281–3288

Sun Y, Chen L, Hou XG, Hou WK, Dong JJ, Sun L, Tang KX, Wang B, Song J, Li H, Wang KX (2007) Differentiation of bone marrow-derived mesenchymal stem cells from diabetic patients into insulin-producing cells in vitro. Chin Med J (Engl) 120:771–776

Tamada H, Nezu R, Matsuo Y, Imamura I, Takagi Y, Okada A (1993) Alanyl glutamine-enriched total parenteral nutrition restores intestinal adaptation after either proximal or distal massive resection in rats. JPEN J Parenter Enteral Nutr 17:236–242

Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, Masteller EL, McDevitt H, Bonyhadi M, Bluestone JA (2004) In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J Exp Med 199:1455–1465

Tarbell KV, Yamazaki S, Steinman RM (2006) The interactions of dendritic cells with antigen-specific, regulatory T cells that suppress autoimmunity. Semin Immunol 18:93–102

Tee CT, Wallis K, Gabe SM (2011) Emerging treatment options for short bowel syndrome: potential role of teduglutide. Clin Exp Gastroenterol 4:189–196

Tian J, Atkinson MA, Clare-Salzler M, Herschenfeld A, Forsthuber T, Lehmann PV, Kaufman DL (1996a) Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. J Exp Med 183:1561–1567

Tian J, Clare-Salzler M, Herschenfeld A, Middleton B, Newman D, Mueller R, Arita S, Evans C, Atkinson MA, Mullen Y, Sarvetnick N, Tobin AJ, Lehmann PV, Kaufman DL (1996b) Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. Nat Med 2:1348–1353

Tisch R, Liblau RS, Yang XD, Liblau P, McDevitt HO (1998) Induction of GAD65-specific regulatory T-cells inhibits ongoing autoimmune diabetes in nonobese diabetic mice. Diabetes 47:894–899

Tisch R, Wang B, Serreze DV (1999) Induction of glutamic acid decarboxylase 65-specific Th2 cells and suppression of autoimmune diabetes at late stages of disease is epitope dependent. J Immunol 163:1178–1187

Tuch BE, Hughes TC, Evans MD (2011) Encapsulated pancreatic progenitors derived from human embryonic stem cells as a therapy for insulin-dependent diabetes. Diabetes Metab Res Rev 27:928–932

Ugrasbul F, Moore WV, Tong PY, Kover KL (2008) Prevention of diabetes: effect of mycophenolate mofetil and anti-CD25 on onset of diabetes in the DRBB rat. Pediatr Diabetes 9:596–601

van Belle TL, Coppieters KT, von Herrath MG (2011) Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiol Rev 91:79–118

Vardanyan M, Parkin E, Gruessner C, Rodriguez Rilo HL (2010) Pancreas vs. islet transplantation: a call on the future. Curr Opin Organ Transplant 15:124–130

Vehik K, Cuthbertson D, Ruhlig H, Schatz DA, Peakman M, Krischer JP (2011) Long-term outcome of individuals treated with oral insulin: diabetes prevention trial-type 1 (DPT-1) oral insulin trial. Diabetes Care 34:1585–1590

Voltarelli JC, Couri CE, Stracieri AB, Oliveira MC, Moraes DA, Pieroni F, Coutinho M, Malmegrim KC, Foss-Freitas MC, Simoes BP, Foss MC, Squiers E, Burt RK (2007) Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 297:1568–1576

von Herrath M, Sanda S, Herold K (2007) Type 1 diabetes as a relapsing-remitting disease? Nat Rev Immunol 7:988–994

Waldmann H, Cobbold S (1998) How do monoclonal antibodies induce tolerance? A role for infectious tolerance? Annu Rev Immunol 16:619–644

Waldron-Lynch F, Herold KC (2009) Advances in Type 1 diabetes therapeutics: immunomodulation and beta-cell salvage. Endocrinol Metab Clin North Am 38:303–317

Waldron-Lynch F, Herold KC (2011) Immunomodulatory therapy to preserve pancreatic beta-cell function in type 1 diabetes. Nat Rev Drug Discov 10:439–452

Wales PW, Nasr A, de Silva N, Yamada J (2010) Human growth hormone and glutamine for patients with short bowel syndrome. Cochrane Database Syst Rev CD006321

Wang TC, Bonner-Weir S, Oates PS, Chulak M, Simon B, Merlino GT, Schmidt EV, Brand SJ (1993) Pancreatic gastrin stimulates islet differentiation of transforming growth factor alpha-induced ductular precursor cells. J Clin Invest 92:1349–1356

Wang RN, Kloppel G, Bouwens L (1995) Duct- to islet-cell differentiation and islet growth in the pancreas of duct-ligated adult rats. Diabetologia 38:1405–1411

Wherrett DK, Bundy B, Becker DJ, DiMeglio LA, Gitelman SE, Goland R, Gottlieb PA, Greenbaum CJ, Herold KC, Marks JB, Monzavi R, Moran A, Orban T, Palmer JP, Raskin P, Rodriguez H, Schatz D, Wilson DM, Krischer JP, Skyler JS (2011) Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: a randomised double-blind trial. Lancet 378:319–327

Wu AJ, Hua H, Munson SH, McDevitt HO (2002) Tumor necrosis factor-alpha regulation of CD4+CD25+ T cell levels in NOD mice. Proc Natl Acad Sci USA 99:12287–12292

Xu G, Stoffers DA, Habener JF, Bonner-Weir S (1999) Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. Diabetes 48:2270–2276

Xu X, D'Hoker J, Stange G, Bonne S, De Leu N, Xiao X, Van de Casteele M, Mellitzer G, Ling Z, Pipeleers D, Bouwens L, Scharfmann R, Gradwohl G, Heimberg H (2008) Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 132:197–207

You S, Leforban B, Garcia C, Bach JF, Bluestone JA, Chatenoud L (2007) Adaptive TGF-beta-dependent regulatory T cells control autoimmune diabetes and are a privileged target of anti-CD3 antibody treatment. Proc Natl Acad Sci USA 104:6335–6340

Yusta B, Huang L, Munroe D, Wolff G, Fantaske R, Sharma S, Demchyshyn L, Asa SL, Drucker DJ (2000) Enteroendocrine localization of GLP-2 receptor expression in humans and rodents. Gastroenterology 119:744–755

Zahr E, Molano RD, Pileggi A, Ichii H, Jose SS, Bocca N, An W, Gonzalez-Quintana J, Fraker C, Ricordi C, Inverardi L (2007) Rapamycin impairs in vivo proliferation of islet beta-cells. Transplantation 84:1576–1583

Zhang ZJ, Davidson L, Eisenbarth G, Weiner HL (1991) Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. Proc Natl Acad Sci USA 88:573–580

Zhang YW, Ding LS, Lai MD (2003) Reg gene family and human diseases. World J Gastroenterol 9:2635–2641

Zhang N, Su D, Qu S, Tse T, Bottino R, Balamurugan AN, Xu J, Bromberg JS, Dong HH (2006) Sirolimus is associated with reduced islet engraftment and impaired beta-cell function. Diabetes 55:2429–2436

Chapter 40
Preclinical Animal Models for Segmental Bone Defect Research and Tissue Engineering

Johannes C. Reichert, Arne Berner, Siamak Saifzadeh, and Dietmar W. Hutmacher

Abstract Commonly applied therapies to achieve bone reconstruction or function are restricted to the transplantation of autografts and allografts, or the implantation of metal devices or ceramic-based implants. Bone grafts generally possess osteoconductive and osteoinductive properties. They are, however, limited in access and availability and harvest is associated with donor site morbidity, hemorrhage, risk of infection, insufficient transplant integration, and graft devitalisation. As a result, recent research focuses on the development of alternative therapeutic concepts. Available literature indicates that bone regeneration has become a focus area in the field of tissue engineering. Hence, a considerable number of research groups and commercial entities work on the development of tissue engineered constructs to aid bone regeneration. However, bench to bedside translations are still infrequent as the process towards approval by regulatory bodies is protracted and cost-intensive. Approval requires both comprehensive *in vitro* and *in vivo* studies necessitating the utilisation of large preclinical animal models. Consequently, to allow comparison between different studies and their outcomes, it is essential to standardize animal models, fixation devices, surgical procedures and methods of taking measurements to produce reliable data pools as a base for further research directions. The following chapter reviews animal models of the weight-bearing lower extremity utilized in the field, which include representations of fracture-healing, segmental bone defects, and fracture non-unions.

J.C. Reichert, M.D., Ph.D. • A. Berner, M.D. • S. Saifzadeh, DVM., Ph.D.
D.W. Hutmacher, Ph.D., M.B.A., M.Sc. (✉)
Institute of Health and Biomedical Innovation, Queensland University of Technology,
60 Musk Avenue, Kelvin Grove, QLD 4059, Australia
e-mail: dietmar.hutmacher@qut.edu.au

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_40,

40.1 Clinical Background

In general, bone displays a high intrinsic regenerative capacity following insult or disease. Therefore, the majority of bone defects and fractures heal spontaneously. Refinements in surgical techniques, implant design and postoperative care have significantly improved treatment outcomes of complex fractures and defects as caused by high energy trauma, disease, developmental deformity, revision surgery, and tumour resection (Perka et al. 2000; Gugala and Gogolewski 2002; den Boer et al. 2003; Komaki et al. 2006; Laurencin et al. 2006; Wildemann et al. 2007). Extensive soft tissue damage, insufficient surgical techniques, infections, and biomechanical instability can, however, lead to formation of large defects with limited intrinsic regenerative potential (Perry 1999). These defects represent a considerable surgical challenge, are associated with high socio-economical costs, and highly influence patients' quality of life. Understanding the factors and microenvironmental cues that favour the formation of such slow or non-healing defects therefore poses a major research challenge (DeCoster et al. 2004; Clements et al. 2008).

Cancellous bone fractures often lead to impaction of bone and consequently defect formation after reduction (den Boer et al. 2003). The tibial diaphysis, which consists of compact bone, however, represents the most common anatomic site for segmental bone defects since soft tissue coverage, especially on the anteromedial surface, is marginal (DeCoster et al. 2004). This both increases the risk of bone loss and complicates treatment (DeCoster et al. 2004).

Presently, the transplantation of bone autografts is considered the "gold standard" treatment to augment or accelerate bone regeneration (Einhorn et al. 1984; Perka et al. 2000; Komaki et al. 2006) (Fig. 40.1). Nevertheless, considerable shortcomings are associated with bone grafting. Graft harvest results in prolonged anaesthesia and requires personnel for graft collection (Bucholz et al. 1989; Gao et al. 1996; Liu et al. 2008). Often, harvested graft amounts are insufficient while donor site accessibility is limited (Stevenson 1998; Blokhuis et al. 2000; Oest et al. 2007; Liu et al. 2008). Persistent pain at donor sites or hemorrhage can occur, and the risk of infection is significantly increased. Once transplanted, donor bone, is associated with a high rate of failure (Sciadini et al. 1997; Blokhuis et al. 2000; den Boer et al. 2002; Liu et al. 2008), which commonly results from incomplete transplant integration and positively correlates with defect size (Gao et al. 1996). Graft devitalisation and subsequent resorption processes can lead to decreased mechanical stability (Younger and Chapman 1989) and consequently compromised bone healing. Vascularised autografts are technically challenging; allografts and xenografts are prone to immune-mediated rejection, graft sequestration and transmission of infectious disease (Taylor et al. 1975; Dell et al. 1985; Gazdag et al. 1995; Puelacher et al. 1996; Chapman et al. 1997; Lindsey et al. 2006; Muscolo et al. 2006; Clements et al. 2008). The high density of cortical bone allografts hinders both sufficient revascularization and cellular invasion from the surrounding host tissue after graft transplantation (Oest et al. 2007). The limited

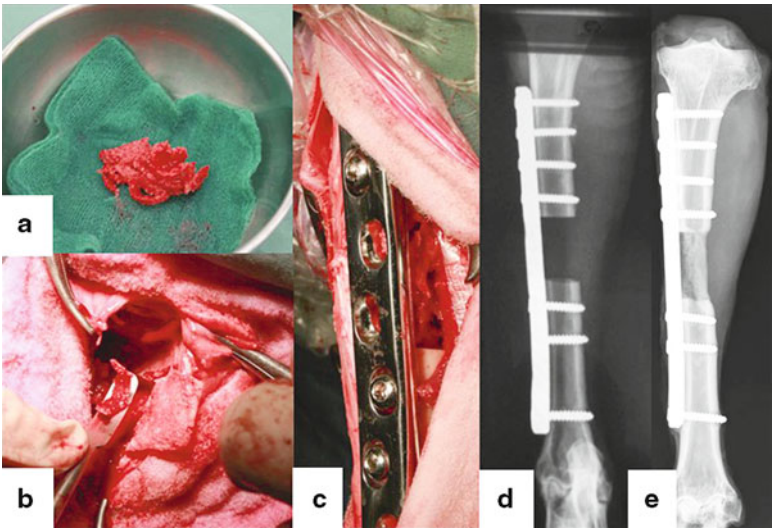


Fig. 40.1 Autologous, cancellous bone graft (**a**) harvested from the iliac crest (**b**) was used to reconstruct a 3 cm critical sized defect in an ovine tibia (**c**, **d**) Defects were stabilized with a 4.5 mm broad dynamic compression plate (Synthes). Twelve weeks after surgery, new bone formation had resulted in solid bony union (**e**)

revascularization and remodelling ability of allografts account for graft failure rates of 25% and complication rates of 30–60% (Cacchioli et al. 2006; Oest et al. 2007). The "Ilizarov technique" aims to circumvent these graft and integration related issues. It is based on osteotomies combined with bone distraction and is successfully applied to treat large bone defects, infected non-unions and limb length discrepancy (Cierny and Zorn 1994). However, this approach is long-lasting, inconvenient for the patient (Goldstrohm et al. 1984; Ilizarov 1989) and recurrent pin track infections and pin loosening are common complications (Lindsey et al. 2006; Gugala et al. 2007).

To avoid the limitations related to the current standard treatments, research interest has recently focused on the use of naturally derived or synthetic bone graft substitutes, and the concept of tissue engineering has emerged as an important alternative approach to regenerate compromised bone. Tissue engineering unites facets of cellular biology, biomechanical engineering, biomaterial sciences and trauma and orthopaedic surgery. It involves the association of cells and/or growth factors with a natural or synthetic scaffold to produce an implantable, three-dimensional construct to support regeneration.

To simulate human *in vivo* conditions and to assess the effects of bone grafts and tissue engineered constructs various large animal models have been developed. Most models, however, are not well described, defined, or standardized, and provide only rudimentary information on the process of model establishment.

40.2 Definition of a Critical-Sized Bone Defect

Experimentally inflicted osseous injuries to study bone repair are postulated to be of dimensions to preclude spontaneous healing (Einhorn 1999). Therefore, the non-regenerative threshold of bone was determined in a variety of research animal models. Critical-sized defects can be defined as "the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal" (Gugala and Gogolewski 1999; Rimondini et al. 2005; Cacchioli et al. 2006) or as a defect which shows less than 10% bony regeneration during the lifetime of an animal (Gugala and Gogolewski 1999).

The minimum size that defines a defect as "critical" is not well understood. Nevertheless, it has been described as a segmental bone deficiency exceeding 2–2.5 times the diameter of the affected bone (Lindsey et al. 2006; Gugala et al. 2007). However, defect healing also depends on the species' phylogenetic scale, anatomic defect location, associated soft tissue, and biomechanical conditions in the affected limb as well as age, metabolic and systemic conditions, and related co-morbidities (Lindsey et al. 2006; Rimondini et al. 2005).

40.3 Large Animal Models in Bone Defect Research

Animal models in bone repair research include models of normal fracture healing, segmental bone defects, and non-unions. With non-unions regular healing processes are compromised in absence of a critical-sized defect site (Tseng et al. 2008). Deficient signalling mechanisms, biomechanical stimuli or cellular responses prevent defect healing. Critical-sized segmental defects, however, do not bridge despite a sufficient biological microenvironment due to the loss of critical amounts of bone substance.

The selection of a specific animal species as a model system requires consideration of multiple factors. The chosen animal model should clearly demonstrate close physiological and pathophysiological analogies with humans regarding the scientific question under investigation. Moreover, it must be manageable to operate and observe a multiplicity of study objects over a relatively short period of time (Schimandle and Boden 1994; Liebschner 2004; Egermann et al. 2005). Further selection criteria include costs for acquisition and care, animal availability, acceptability to society, tolerance to captivity and ease of housing (Pearce et al. 2007).

40.3.1 Dogs

A number of publications have described dogs as a suitable model for research related to human orthopaedic conditions (Martini et al. 2001). In regards to bone weight, density and bone material constituents such as hydroxyproline, extractable proteins, IGF-1 content, organic, inorganic and water fraction, dogs are the closest

to humans although clear differences in bone microstructure and remodelling have been described (Gong et al. 1964; Aerssens et al. 1998). While the secondary structure of human bone is predominantly organized in osteones, the osteonal bone structure in dogs is limited to the core of cortical bone, whereas in areas adjoining the periosteum and endosteum mainly laminar bone is found as characteristic for large, fast-growing animals (Wang et al. 1998). It has been reported that generally, higher rates of trabecular and cortical bone turnover can be observed in dogs compared to humans (Bloebaum et al. 1993) and differences in loads acting on the bone as a result of the dog's quadrupedal gait must be taken into consideration as well. A review article by Neyt states that between 1991 and 1995 11% of musculoskeletal research was undertaken in dogs, results that are confirmed by Martini et al. who find that between 1970 and 2001 9% of orthopaedic and trauma related research used dogs as animal models for orthopaedic and trauma related research (Neyt et al. 1998; Martini et al. 2001). Recently, the use of dogs as experimental models has significantly decreased mainly due to ethical issues (O'Loughlin et al. 2008).

40.3.2 Sheep and Goats

Mature sheep and goats possess a bodyweight comparable to adult humans and long bone dimensions enabling the use of human implants (Newman et al. 1995). The mechanical loading environment in sheep is well understood. The loads, forces and moments acting within the hind limb bones, are approximately half of that determined for humans during normal walking (Taylor et al. 2005, 2006). Since no major differences in mineral composition (Ravaglioli et al. 1996) are evident and both metabolic and bone remodelling rates are akin to humans (Anderson et al. 1999), sheep are considered a valuable model for human bone turnover and remodelling activity (den Boer et al. 1999). Bone histology, however, reveals some differences in bone structure between sheep and humans. In sheep, bone consists principally of primary bone (de Kleer 2006) in contrast to the largely secondary, haversian bone composition in humans (Eitel et al. 1981); furthermore, secondary, osteonal remodelling in sheep does not take place until an average age of 7–9 years (Newman et al. 1995). A significantly higher trabecular bone density and greater bone strength was described for mature sheep when compared to humans, the trabecular bone in immature sheep, however, is weaker, has a lower stiffness and density, a higher flexibility (higher collagen content) (Nafei et al. 2000), and shows a comparable bone healing potential and tibial blood supply (Dai et al. 2005).

40.3.3 Pigs

In a variety of study designs, pigs are considered the animal of choice and were – despite their denser trabecular network (Mosekilde et al. 1993) – described as a highly representative model of human bone regeneration processes in respect to

anatomical and morphological features, healing capacity and remodelling, bone mineral density and concentration (Aerssens et al. 1998; Thorwarth et al. 2005). However, pigs are often neglected in favour of sheep and goats as the handling of pigs is rather intricate (Newman et al. 1995). Furthermore, the short length of the tibiae and femora in pigs may bring about the need for special bone/fracture fixation devices, as implants designed for humans cannot be used. For studies assessing orthopaedic knee and hip implants, however, pigs or pig bones are considered a standard model.

40.4 Femoral Fracture Models

40.4.1 Sheep and Goats

With the objective to develop a single-channel telemetric intramedullary nail that measures anterior-posterior bending strains and to determine whether these forces decrease sigmoidally when normalized to the ground reaction force during fracture healing, Wilson et al. (2009) stabilized a transverse mid-shaft femoral osteotomy (1 mm) using a customized TriGen intramedullary nail incorporating a strain gauge in the anterior–posterior plane. Fourteen skeletally mature sheep (2–3 years old) were treated in two pilot studies (n = 3/pilot) and a pivotal study (n = 8). Three animals, however, had to be excluded. Static strain measurements were acquired at approximately 130 Hz during leg stance. *In vivo* gait analysis was carried out weekly to assess ground reaction forces and fortnightly x-rays to assess stability and fracture healing. Animals were euthanized 12 weeks postoperatively. Callus formation was assessed by microcomputed tomography and histomorphometry. The degree of load shared between bone and the nail was determined post mortem by three-point bending. Study results indicated a significant preload generated during implantation, most notably during placement of the four interlocking screws and by the action of attached soft tissues. Eight animals showed evidence of bone healing by x-ray, microcomputed tomography, and histology. However, a reduction in implant load was only observed with two of the eight. The degree of load sharing observed *in vivo* in these animals (50–75%) compared favourably with *in vitro* observations (approximately 50%). In the non-healing ambulating animals, nail forces did not change over time. Three-point bending tests carried out on "healed" femora suggested that load sharing between the bone and nail could be detected more easily in the absence of soft tissues. No clear correlation between implant strain and fracture healing was observed using the single-channel system when subjected to one external loading regime (leg stance phase). However, *ex vivo* biomechanical testing demonstrated that load share changes could be detected when loads were directly applied to the bone in the absence of muscle and ligament forces. It was concluded that these data emphasize the need to fully characterize the complex biomechanical environment of the limb to determine the load changes resulting from fracture healing.

Gray et al. conducted as study to compare the physiologic effects of external femoral fixation with those of intramedullary stabilization over the first 24 h after femoral fracture (Gray et al. 2009). Under terminal anaesthesia, bilateral high-energy femoral fractures and hypovolemic shock were produced using a pneumatic actuator. Twenty-four sheep were randomized into four groups and monitored for 24 h. Group 1 – control, Group 2 – trauma only, Group 3 – trauma and external fixation, and Group 4 – trauma and reamed intramedullary nailing. Outcome measures included the pulmonary embolic load (transesophageal echocardiography), metabolic base excess, plasma coagulation markers, and polymorphonuclear cell counts obtained from bronchoalveolar lavage samples. It was found that the total embolic load was significantly higher in the intramedullary nailing group. All trauma groups had a significant increase in prothrombin times with a fall in antithrombin III and fibrinogen levels. However, the type of fracture stabilization used did not significantly affect any of the other outcome measurements.

Another study using an ovine femoral fracture model assessed the influence of driving speed and revolution rate per minute of two reamers on femoral intramedullary pressure increases and fat intravasation (Mousavi et al. 2002). The AO and Howmedica reamers were tested in four groups with different combinations of driving speed and revolution rate per minute in both femora in a sheep model. The 24 animals were exposed to hemorrhagic shock after mid-shaft osteotomy and were resuscitated before reaming of both femoral shafts. Controlled reaming was performed at 15 and 50 mm/s driving speed with 150 and 450 rpm. Transesophageal echocardiography, Gurd tests, and a piezoelectric gauge measured fat intravasation and the intramedullary pressure. Low driving speed and high revolutions per minute with the smaller cored reamer led to lower intramedullary pressure changes. The same reaming parameters led to greater pulmonary stress during surgery of the second side. The authors summarized that reaming with a smaller cored reamer and modified reaming parameters leads to a lower increase in intramedullary pressure and reduces the amount of fat intravasation. Primary reamed intramedullary nailing should therefore be done after resuscitation at a low driving speed and high revolutions per minute with a smaller cored reamer to minimize the risk of pulmonary dysfunction.

The role and long-term degradation kinetics of synthetic polymers used for fracture fixation is still unclear. Therefore, van der Elst et al. initiated a research project to investigate biodegradable interlocking nails in a long-term setting (van der Elst et al. 1999). In 21 female sheep a complete mid-shaft osteotomy of the left femur was performed to mimic a fracture of the femoral shaft. For fixation, an intramedullary stainless-steel interlocking nail, a PLA rod or a PLA/PGA rod was used. Thirty months after implantation the histological sections of all groups were examined. In contrast to most reports the degradation rate of both polymers was much slower than suggested by the manufacturer (24 months). The response of the surrounding tissue was more pronounced than anticipated. It was summarised that this foreign body reaction together with the slow degradation kinetics of the polymers may imply certain risks for device rejection and may negatively influence clinical outcomes.

A minimally invasive approach to create a multifragmental fracture in the sheep femur (classification by the Association for the Study of Internal Fixation, AO type 32-C), in which the bone was weakened by two short, transverse anterior osteotomies and bi-cortical drill holes created through small incisions, has recently been described by Wullschleger et al. The insertion of two chisels and one blade bar were then used to initiate cracks connecting both the osteotomies and the drill holes, thereby creating a standardized multifragmental fracture. A minimally invasive approach for fracture fixation (MIPO) was compared to the traditional open approach (n = 8). In both groups, fractures were stabilized with a 4.5 mm narrow 7-hole LC-LCP (Synthes) (unpublished data).

40.4.2 Dogs

The dog is one of the most frequently used large animal species for musculoskeletal and dental research (Neyt et al. 1998; Martini et al. 2001). The use of dogs as research animals varies greatly depending on the societal acceptance. In the 1980s, a number of biomaterials were tested in canine models for their suitability to serve as stabilization systems of femoral fractures. Kettunen et al. used a femoral fracture model in dogs to test the stability of newly developed carbon fibre-reinforced liquid crystalline polymers (LCF/CF) (Kettunen et al. 1999). They have operated 14 Beagle dogs with a follow up of 1 and 2 years, respectively. The osteotomy healed with a strong callus formation in all dogs after 12 weeks and there was no significant loss of length in the femur compared to the contralateral control femur. The implants fabricated from carbon fibre-reinforced liquid crystalline polymer showed sufficient mechanical properties and biocompatibility for intramedullary use in load-bearing applications in the 2-year follow-up.

The effects and applicability of an intramedullary self-reinforced polyglycolic acid, a self-reinforced poly-L-lactic acid and a metallic rod in the fixation of growing bones in a femoral shaft osteotomy were analysed by Miettinen et al. (1992). Fifteen beagle dogs, 12 weeks of age, were used. In all animals a solid union of the osteotomy without secondary displacement was seen radiographically 6 weeks after surgery. None of the three different biomaterials used in this experiment caused a significant disturbance to the longitudinal growth of the operated femur. Jockisch et al. evaluated a 30% chopped-carbon-fiber-reinforced poly(etheretherketone) (CFRPEEK) as a potential material for the use as a fracture fixation plate (Jockisch et al. 1992). The plates were implanted as internal fixation devices for femoral mid-shaft osteotomies in ten beagle dogs for 8 and 16 weeks. They were effective in promoting fracture healing; however, a non-specific foreign body reaction to the plates was observed. The use of short carbon fibre reinforced thermoplastic plates for internal fixation of canine femoral transverse mid-shaft fractures was tested by Gillett et al. (1985). After 8 and 12 weeks, they noted a moderate inflammatory reaction while the material allowed sufficient support for the healing fracture without preventing the remodelling process.

40.4.3 Pigs

The surgical treatment of fracture models or bone defects of the femur in pigs is rather difficult due to extensive soft tissue coverage and it's short length. Therefore, most studies focus on the metaphyseal area of the pig femur or the knee joint (Jiang et al. 2007; Gotterbarm et al. 2008). Kleinmann et al. analysed the image optimization and dose reduction with computed radiography for the detection of simulated inflicted metaphyseal fractures in a fetal pig model (Kleinman et al. 2008). They suggest that computed radiography can replace screen-film imaging in the detection of classic metaphyseal fractures and may permit dose reduction.

40.5 Femoral Segmental Defect Models

40.5.1 Sheep and Goats

A mid-diaphyseal, 2.5 cm long osteoperiosteal segmental defect stabilized by plate fixation was created in the right femur of 17 sheep (Gerhart et al. 1993). Four treatment groups were included: Group I, no implant; Group II, inactive bone matrix; Group III, recombinant human bone morphogenetic protein (rhBMP-2) mixed with inactive bone matrix; and Group IV, autologous bone graft. Three animals had early failure of fixation, and the remaining 14 were evaluated at 3 months after implantation. Radiographs showed bony union of all defects treated with rhBMP-2 (six) and a lack of bony union in the negative-control groups treated with no implant (three) and inactive bone matrix without BMP (three). Both defects treated with autograft healed. New bone formation in the defect sites treated with rhBMP-2 first appeared 1 month after implantation and had a mean bending strength (expressed as a percentage of the contralateral femur) of 91% ± 59% (mean ± standard deviation) for defects treated with BMP-2, 77% ± 34% for autograft, 9% ± 8% for no implant, and 11% ± 7% for inactive matrix without BMP. Three sheep treated with rhBMP-2 had their fixation plates removed at 4 months and were followed for 1 year. Their bone defect sites remained solidly healed 1 year after the initial operation.

Mid-diaphyseal 2.5 cm segmental defects in the right femora of 12 sheep were stabilized with stainless steel plates and treated with (1) 2 mg rhBMP-2 and poly[D,L-(lactide-co-glycolide)] bioerodible polymer with autologous blood (n = 7), (2) 4 mg rhBMP-2 and poly[D,L-(lactide-co-glycolide)] and blood (n = 3), or (3) poly[D,L-(lactide-co-glycolide)] and blood only (n = 2) (Kirker-Head et al. 1998). Bone healing was evaluated for 1 year using clinical, radiographic, gross pathological and histological techniques. Union occurred in three sheep in Group 1, two in Group 2, and none in Group 3. In healing defects, new bone was first visible radiographically between weeks two and six after implantation; new bone mineral content equalled that of the intact femur not surgically treated by week 16; recanalization of the medullary cavity approached completion at week 52; and at necropsy the surgically

treated femora were rigidly healed, the poly[D,L-(lactide-co-glycolide)] was resorbed completely, and woven and lamellar bone bridged the defect site. In two Group 1 sheep euthanized at weeks two and six, polymer particles were permeated by occasional multinucleated giant cells. Some plasma cells, lymphocytes, and neutrophils were present locally. The poly[D,L-(lactide-co-glycolide)] tended to fragment during surgical implantation. Despite these observations, the recombinant human bone morphogenetic protein 2/poly[D,L-(lactide-co-glycolide)] implant was able to heal large segmental bone defects in this model.

Zhu et al. tissue engineered bone using osteogenically induced MSCs (Zhu et al. 2006) isolated from bone marrow aspirates. Coral based scaffolds were used to facilitate cell administration. A 25 mm long defect was created in the mid-diaphyseal region of the right femur in a total of 20 goats and fixed using an intramedullary nail. Control animals received coral cylinders only ($n = 10$). In the experimental group, bony union was observed radiographically at 4 months, and engineered bone was further remodelled into cortical bone at 8 months. H&E staining demonstrated that trabecular bone at 4 months and irregular osteon formation at 8 months. In respect to bending strength and stiffness, the tissue-engineered bone was not significantly different from the contralateral paired control ($p > 0.05$). In contrast, the coral cylinders of the control group showed considerably less bone formation. Almost complete resorption of the carrier had occurred after 2 months; only a small amount of residual coral particles surrounded by fibrous tissue was evident at 4 months whereas the residues had disappeared at 8 months.

In a follow up study, the feasibility to use bone marrow stromal cells (BMSCs) infected with an adenoviral vector containing the BMP-7 gene (AdBMP7) to enhance bone regeneration was investigated. Defects were reconstructed with AdBMP7-infected BMSCs/coral or non-infected BMSCs/coral. The results suggested that cellular overexpression of BMP-7 additionally stimulates bone healing (Zhu et al. 2010).

A recently proposed one-stage bone transport surgical procedure exploits the intrinsic osteogenic potential of the periosteum while providing mechanical stability through intramedullary nailing. Knothe Tate et al. therefore assessed the efficacy of this technique to bridge long bone defects in a single stage (Knothe Tate et al. 2007). With use of an ovine femoral model, an *in situ* periosteal sleeve was elevated circumferentially from healthy diaphyseal bone, which was osteotomized and transported over an intramedullary nail into a 2.54 cm critical-sized diaphyseal defect. The defect bridging and bone regenerating capacity of the procedure were tested in five groups of seven animals each, which were defined by the absence (Group 1; control) or presence of the periosteal sleeve alone (Group 2), bone graft within the periosteal sleeve (Groups 3 and 5), as well as retention of adherent, vascularized cortical bone chips on the periosteal sleeve with or without bone graft (Groups 4 and 5). The efficacy of the procedure was assessed qualitatively and quantitatively. At 16 weeks, osseous bridging of the defect was observed in all 28 experimental sheep in which the periosteal sleeve was retained; the defect persisted in the remaining seven control sheep. Among the experimental Groups 2–5, significant differences were observed in the density of the regenerated bone tissue; the two groups in which

vascularized bone chips adhered to the inner surface of the periosteal sleeve (Groups 4 and 5) showed a higher mean bone density in the defect zone ($p<0.02$) than the other groups. In these two groups with the highest bone density, the addition of bone graft was associated with a significantly lower callus density than that observed without bone graft ($p<0.05$). The volume of regenerate bone ($p<0.02$) was significantly greater in the groups in which the periosteal sleeve was retained than in the control group. Among the experimental groups (Groups 2–5), however, with the numbers studied, no significant differences in the volume of regenerate bone could be attributed to the inclusion of bone graft within the sleeve or to vascularized bone chips remaining adherent to the periosteum. The authors concluded that the novel surgical procedure was shown to be effective in bridging a critical-sized defect in an ovine femoral model. Vascularized bone chips adherent to the inner surface of the periosteal sleeve, without the addition of morselized cancellous bone graft within the sleeve, provide not only a comparable volume of regenerate bone and composite tissue (callus and bone) but also a superior density of regenerate bone compared to that after the addition of bone graft.

Using the same animal model, the authors furthermore tested the hypothesis that directional delivery of endogenous periosteal factors enhances bone defect healing (Knothe Tate et al. 2011). Defects ($n=5$ per group) were treated with an isotropic elastomeric silicone membrane, membrane plus bovine collagen, silicone with collagen and periosteal cells or silicone membranes combined with autologous periosteal strips. Micro-computed tomography showed that bone defects enveloped by a substitute periosteum exhibited superior bony bridging compared to those treated with isotropic silicone membrane controls. Greatest tissue generation and defect bridging was observed when autologous periosteal transplant strips were transplanted. In summary, the authors drew the conclusion that periosteum derived cells, besides other factors intrinsic to periosteum, play a key role for infilling of critical sized defects.

Currently available synthetic void fillers are indicated for bony voids or gaps that are not intrinsic to the stability of the structure. Jax TCP (tricalcium phosphate) is an osteoconductive bioceramic fabricated into 4 mm granules with a unique interlocking form, promoting structural integrity while allowing bone ingrowth. Field et al. (2009) conducted a study to assess bone ingrowth using a large, critically sized, femoral defect. A 5 cm segmental ostectomy was created in the mid-diaphysis of 16 adult ovine femora. A stainless steel intramedullary nail was introduced and locked with two proximal and two distal fully-threaded locking screws. Each defect was surrounded with a resorbable macroporous poly(L-lactide-co-D,L) lactide mesh acting as graft containment. Treatment groups ($n=4$) were as follows: (1) Empty defect; (2) Morselized cortical bone; (3) Cortical strut; (4) Jax TCP. Serial radiographs were taken postoperatively and at 2, 4 and 6 months. Femora retrieved at necropsy (6 months) underwent computed tomography for volumetric analysis followed by histological assessment of the biological response. Little bone was apparent in the empty defect group, whereas significant bone was evident in both autograft groups and the Jax TCP group. Three-dimensional CT reconstructions and volumetric analysis were in close agreement

with the radiographic findings. Jax TCP bone graft substitute has been proven to be effective in the healing of a large, critically sized, contained segmental defect. The healing observed was superior to that of cortical struts and the new bone laid down had similar radio-opacity to autograft.

Lian et al. investigated the suitability of bone marrow stromal cells (BMSCs) transfected with an adenoviral vector containing the gene encoding for BMP-7 (AdBMP7) to enhance bone regeneration in a critically sized femoral defect in the goat model (Lian et al. 2009). The defects of 25 mm length were filled with AdBMP7-infected BMSCs/coral (BMP-7 group) or non-infected BMSCs/coral (control group) implants, respectively, and stabilized with an internal interlocking IM nail. Bridging of the segmental defects was evaluated by monthly radiographs, and confirmed by biomechanical three point bending tests. Extensive callus formation was found in the BMP-7 group, and intramedullary nails could be removed 3 months after implantation to allow the regenerated bone in the defect to remodel. In the control group, the nails were removed after 6 months. Biomechanical testing revealed restoration of the mechanical properties at 5 months in the BMP-7 group, however not until 8 months in the control group.

In yet another study, Nzair et al. (2009) evaluated a triphasic ceramic (calcium silicate, hydroxyapatite and tricalcium phosphate)-coated hydroxyapatite (HASi) as a bone substitute for the repair of segmental defects (2 cm) created in a goat femur model. Three experimental goat femur implant groups – (a) bare HASi, (b) osteogenicly induced goat bone marrow-derived mesenchymal stem cells cultured HASi (HASi+C) and (c) osteogenicly induced goat bone marrow-derived mesenchymal stem cells cultured HASi+platelet rich plasma (HASi+CP) – were included and the influence on defect healing was assessed. In all groups, the material united with the host bone without any inflammation and an osseous callus formed around the implant. A remarkable difference between the groups appeared in the mid region of the defect. In bare HASi groups, numerous osteoblast-like cells could be seen together with parts of the biomaterial. However, in HASi+C and HASi+CP, about 60–70% of that area was occupied by woven bone. The interconnected porous nature (50–500 μm), together with the chemical composition of the HASi, facilitated the degradation of HASi, thereby opening up void space allowing for cellular ingrowth and bone regeneration. The combination of HASi with cells and PRP was advantageous promoting the expression of osteoinductive proteins, leading to faster bone regeneration and material degradation. Based on these results, it was concluded that bare HASi can aid in bone regeneration but, with the combination of cells and PRP, the sequence of healing events occurs much faster in large segmental bone defects in weight-bearing areas in goats. However, the study included two animals per group only and no quantitative analysis of bone formation, mechanical properties and no statistical analysis were performed.

Bullens et al. studied whether a static or dynamic mode of nail fixation influences the healing of segmental defect reconstructions in long bones. Femoral 3.5 cm defects were created in skeletally mature milk goats and reconstructed using a cage filled with morselized allograft mixed with hydroxyapatite (Ostim). All defects were stabilised with intramedullary nails. The nails were either locked statically

(n = 6), or in a dynamic mode (n = 6). It was hypothesized that nail dynamisation would stimulate bone healing. The torsional strength determined after sacrifice and normalized to the contralateral femora was 74.8 ± 17.5% (static) and 73.0 ± 13.4% (dynamic) after 6 months. Overall, it was concluded that that defect healing mediated by impacted morselized grafts in a cage is not significantly influenced by the mode of nail fixation (Bullens et al. 2010).

40.5.2 Dogs

A well established procedure for the treatment of defects in long bones is the Ilizarov technique (Ilizarov and Gracheva 1971). Pablos et al. treated large segmental femoral bone defects by bone transport with monolateral external distractors (de Pablos et al. 1994). Segmental bone defects of 4–5 cm length were created in the femoral bone of five male mongrel dogs. The distraction was started at the day after surgery with a rate of 1 mm/day. Results were evaluated on the basis of radiography, computed tomographic (CT) and histologic analyses 20 days after surgery, at the end of distraction and 4-month postoperatively. After 4 months, there was newly formed bone tissue with an appearance similar to normal diaphyseal bone. The efficacy of cylindrical titanium mesh cages (CTMC) for the reconstruction of a critical sized segmental femoral defect in a canine model was analysed by Lindsey et al. (2006). A 3 cm mid-diaphyseal segmental defect was created in the femur of 21 adult dogs and four experimental groups were included. The cylindric titanium mesh cage was packed and surrounded with a standard volume of morselized canine cancellous allograft and canine demineralised bone matrix. The limbs were stabilized using a reamed intramedullary nail. In three groups the defects were CTMC reconstructed and the follow up was 6, 12 and 18 weeks. The defects in the control group were simply stabilized with the nail. With CTMC bone continuity was successfully restored. Takigami et al. focused on the effect by implantation of osteogenic protein-1 in addition to autogenous bone marrow cells in a canine femur defect model (Takigami et al. 2007). Brodke et al. analyzed the healing efficacy of demineralised bone and cancellous chips (DBM-CC) enriched with osteoprogenitor cells (Brodke et al. 2006). All grafts were transplanted unilaterally in a 2.1 cm long osteoperiosteal critical-sized femoral defect. The results demonstrated that SCR-enriched DBM-CC was equivalent to autografts.

40.5.3 Pigs

Critical-sized defects in the femoral bone of pigs are difficult to establish and the number of publications working with such a model is accordingly low. Consequently, predominantly osteochondral defect models of the femoral condyles are described

in the literature. Kilian et al. analysed the cellular activity in the early phase of biodegradation and bone healing of bone substitutes loaded with platelet derived factors (PLF) (Kilian et al. 2007). A cylindric bone defect of 8.9 mm diameter was created in the distal femur condyle of 20 miniature pigs. After 20 days, histomorphometry of new bone formation and of biodegradation of the hydroxyapatite material was performed. In summary, PLF stimulated HA degradation and showed a positive effect on osteogenesis in the early stage of bone healing. Schnettler et al. evaluated angiogenesis, bone formation, and bone ingrowth in response to osteoinductive implants consisting of bovine-derived hydroxyapatite (HA) ceramics either uncoated or coated with basic fibroblast growth factor (bFGF) in cylindrical subchondral defects of the femur of 24 miniature pigs (Schnettler et al. 2003). Fluorochrome labelled histological analysis, histomorphometry, and scanning electron microscopy were performed after 42 and 82 days. The results showed comparable results of bFGF-coated HA implants and autogenous grafts regarding angiogenesis, bone synthesis and bone ingrowth.

40.6 Tibial Fracture Models

Animal fracture models have been widely investigated to identify and further characterize physiological and pathophysiological processes of fracture healing of long bones. One of the most important elements in the study of fracture healing or fixation is the establishment of standardized methods to create reproducible fractures. Although a substantial number of articles on fracture models in animals and treatment options have been described over the last decades, only few publications describe the actual infliction of a fracture by trauma rather than the creation of a bony defect <3 mm size by osteotomy, which is generally accepted as an alternative since it is less problematic to standardize.

40.6.1 Sheep and Goats

As previously mentioned, mature sheep and goats possess a bodyweight similar to adult humans, show no major differences in bone mineral composition with similar metabolic and bone remodelling rates, and therefore are considered a valuable model for human bone turnover and remodelling activity often used in fracture research. In the period between 1990 and 2001, sheep as an animal model were used in 9–12% of orthopaedic research, compared to only 5% between 1980 and 1989 (Martini et al. 2001). Over the last 10 years numbers of studies utilizing sheep and goats as animal models have even increased to 11–15% (O'Loughlin et al. 2008).

To compare the effects of reamed versus unreamed locked intramedullary nailing on cortical bone blood flow Schemitsch et al. created a standardized spiral fracture

by three-point bending with torsion in a fractured sheep tibia model (Schemitsch et al. 1994, 1996), a method also described by Tepic et al. (1997) to establish a standardized oblique fracture in sheep tibiae in order to compare healing in fractures stabilized with either a conventional dynamic compression plate (DCP) or an internal point contact fixator (PC-Fix).

The significance of postoperative mechanical stability for bony repair of a comminuted fracture was investigated in a sheep study comparing four commonly applied operative methods of stabilizing fractures. In this study, a triple-wedge osteotomy of the right sheep tibia was used as a fracture model (Heitemeyer et al. 1990). Using a standard osteotomy of the ovine tibia stabilised by an external skeletal fixator, Goodship et al. elucidated the influence of fixator frame stiffness on bone healing rates (Goodship et al. 1993). Wallace et al. (1995) used a similar model to investigate serum angiogenic factor levels after tibial fracture. Likewise, transverse mid-diaphyseal osteotomies with an interfragmentary gap of 3 mm, as an experimental fracture model in sheep, were used to assess fracture repair processes (Augat et al. 1997, 2003; Schell et al. 2005; Epari et al. 2006). To validate the principle of external fixation dynamization in order to accelerate mineralized callus formation by *in vivo* measurements of callus stiffness, transverse fractures with an interfragmentary gap of 3 mm width were created in the mid third of the tibial diaphysis (Hente et al. 1999). Hantes et al. investigated the effect of transosseous application of low-intensity ultrasound on fracture healing in a mid-shaft osteotomy sheep model (Hantes et al. 2004). Epari et al. were the first authors to report on the pressure, oxygen tension and temperature in the early phase of callus tissue formation of six Merino-mix sheep that underwent a tibial osteotomy to model fracture conditions (Epari et al. 2008). In this study, the tibia was stabilized with a standard mono-lateral external fixator. It was found that the maximum pressure during gait increased from 3 to 7 days. During the same interval, there was no change in the peak ground reaction force or in the interfragmentary movement. Oxygen tension in the haematoma was initially high post-op and decreased steadily over the first 5 days. The temperature increased over the first 4 days before reaching a plateau on day 4.

Mechanical strain during callus distraction is known to stimulate osteogenesis and it is so far unclear whether this stimulus can enhance the healing of a fracture without affecting bone length. Just recently, Claes et al., reported that a slow temporary distraction and compression of a diaphyseal osteotomy accelerates fracture healing (Claes et al. 2008) in a mid-diaphyseal osteotomy fracture model of the right tibia in sheep, stabilized by external fixation.

Lu et al. conducted a study to compare bone healing of tibial osteotomies stabilized with an fixation system consisting of Nitinol wire braids and hardened steel rods combined with polymethylmethacrylate bone cement (Braid system) with an interlocking intramedullary (IM) nail fixation in an ovine model (Lu et al. 2009)(Fig. 40.2). For biomechanical *in vitro* studies, a middiaphyseal, transverse osteotomy was performed in the right tibia of adult female sheep. The bones were randomly assigned to the Braid system or IM nail (n = 5). The left tibiae were used as paired controls. The torsional stiffness of the constructs was determined, showed,

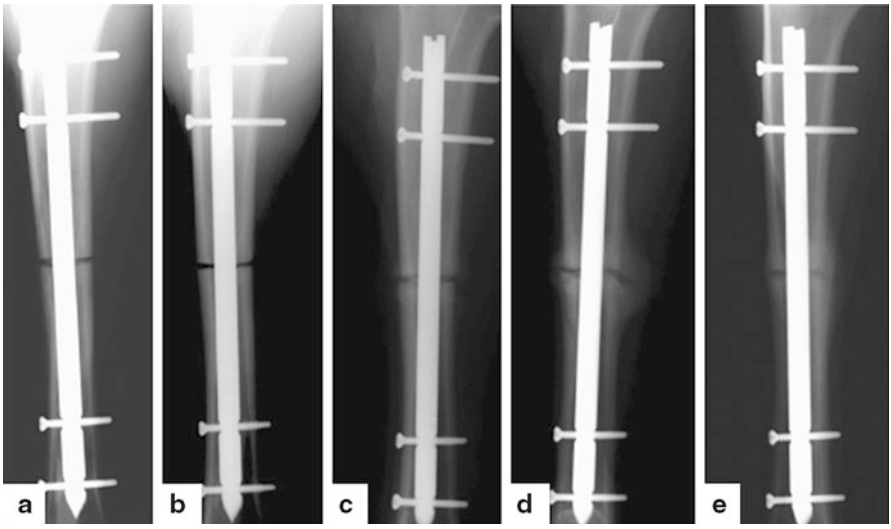


Fig. 40.2 Transverse tibial osteotomy in a sheep tibia stabilized with a intramedullary nail and two locking screws each proximally and distally 0, 2, 4, 8, and 12 weeks after surgery

however, no significant difference. A following *in vivo* study in 12 sheep compared the mechanical properties of healing bone at 12 weeks. While the operative time for the Braid system group was significantly shorter than the IM nail group, no significant differences in maximum torque and torsional stiffness between IM nail and Braid system groups nor significant radiographic or histologic differences between the groups were found.

After bone fracture, a sequence of well orchestrated cellular events lead to the formation of different tissue types, which form the basis for the process of secondary bone healing. Although these tissues have been quantified by histology, their material properties are not well understood. Thus, Manjubala et al. tried to correlate the spatial and temporal variations in the mineral content and the nanoindentation modulus of the callus formed via intramembranous ossification over the course of bone healing (Manjubala et al. 2009). Mid-shaft tibial samples from a sheep osteotomy model at time points of 2, 3, 6 and 9 weeks were employed. PMMA embedded blocks were used for quantitative back scattered electron imaging and nanoindentation of the newly formed periosteal callus near the cortex. The resulting indentation modulus maps show the heterogeneity in the modulus in the selected regions of the callus. The indentation modulus of the embedded callus was about 6 GPa at the early stage. At later stages of mineralization, the average indentation modulus reached 14 GPa. There was a slight decrease in average indentation modulus in regions distant to the cortex, probably due to remodelling of the peripheral callus. The spatial and temporal distribution of mineral content in the callus tissue also illustrated the ongoing remodelling processes observed by histological analysis. Most interestingly the average indentation modulus, even at 9 weeks,

remained as low as 13 GPa, which amounts for about 60% of the modulus for cortical sheep bone. The decreased indentation modulus in the callus compared to cortex was attributed to the lower average mineral content and to the properties of the organic matrix, which differ from normal bone.

Starr et al. assessed the effects of hemorrhagic shock on fracture healing in a closed large animal fracture model (Starr et al. 2002). Standardized bilateral closed mid-shaft tibia fractures were created in eight skeletally mature male goats. The goats were randomized to a hemorrhage, shock and resuscitation group (shock group), or a control group. Hemorrhagic shock was induced in the four goats of the shock group. The shock state was maintained for 30 min. The remaining four goats were used as a control group. All fractures were stabilized with a standardized external fixator. One goat of the shock group became agitated upon emergence from anaesthesia and dislodged two of his external fixator pins. This animal was not included in any further analyses. One goat that would have been entered into the control group was then switched to the shock group, leaving four goats in the shock group and three in the control group. One goat in the shock group developed a non-union of the left tibia fracture. This non-union occurred because of pin loosening in the distal tibia. The non-united bone was excluded from further analysis. Hemorrhage uniformly resulted in shock. Radiographic analysis however showed no apparent differences in healing between groups. With the exception of the non-union, all tibiae were healing uneventfully, resulting in radiographic unions. Biomechanical testing showed no statistical difference between the shock and control groups in regards to maximum torque ($p = 0.95$), stiffness ($p = 0.64$), and energy absorbed at failure ($p = 0.91$). Histomorphologic results revealed no differences between groups. Shock did not appear to influence bone formation rate or callus remodelling. No evidence of osteocyte necrosis was observed. Therefore, it was concluded that transient haemorrhagic shock does not adversely affect closed tibia fracture healing in a goat model.

As little is known about the effectiveness of osteoinductive proteins such as osteogenic protein-1 (OP-1) in stimulating fracture healing, Blokhuis et al. investigated the biomechanical and histological aspects of fracture healing after an injection of OP-1 into a fracture gap (Blokhuis et al. 2001). A closed fracture was created in the left tibia of 40 goats and stabilized with an external fixator. The animals were assigned to four different groups: no injection, injection of 1 mg OP-1, injection of 1 mg OP-1 with collagenous carrier material, and injection of carrier material alone. Twenty-one animals were sacrificed after 2 weeks and 19 after 4 weeks. Biomechanical testing was perfomed on both explanted tibiae. Four longitudinal samples of the fracture were sawn, processed for histology, and examined by two observers. Biomechanical evaluation showed a higher stiffness and strength at 2 weeks after injection of OP-1. Histological evaluation showed normal fracture healing patterns in all animals without adverse effects of the given injections. It was concluded that fracture healing can be accelerated with a single injection of OP-1, eventually resulting in normal bone healing.

In a similar study, Welch et al. studied the effects of rhBMP-2 in an absorbable collagen sponge (ACS) on bone healing in a large animal tibial fracture model

(Welch et al. 1998). Bilateral closed tibial fractures were created in 16 skeletally mature goats and reduced and stabilized using external fixation. In each animal, one tibia received the study device (0.86 mg of rhBMP-2/ACS or buffer/ACS), the contralateral fracture served as control. The device was implanted as a folded onlay or wrapped circumferentially around the fracture. Six weeks following fracture, the animals were sacrificed and the tibiae harvested for torsional testing and histomorphologic evaluation. Radiographs indicated increased callus formation at 3 weeks in the rhBMP-2/ACS treated tibiae. At 6 weeks, the rhBMP-2/ACS wrapped fractures had superior radiographic healing scores compared with buffer groups and controls. The rhBMP-2/ACS produced a significant increase in torsional toughness ($p=0.02$), and trends of increased torsional strength and stiffness ($p=0.09$) compared with fracture controls. The device placed in a wrapped fashion around the fracture produced significantly tougher callus ($p=0.02$) compared with the onlay application. Total callus new bone volume was significantly increased ($p=0.02$) in the rhBMP-2/ACS fractures compared with buffer groups and controls regardless of the method of device application. The rhBMP-2/ACS did not alter the timing of onset of periosteal/endosteal callus formation compared with controls. Neither the mineral apposition rates nor bone formation rates were affected by rhBMP-2/ACS treatment. The increased callus volume associated with rhBMP-2 treatment produced only moderate increases in strength and stiffness.

To investigate the effect of the Reamer/Irrigator/Aspirator (RIA, Synthes) when nailing intramedullary, Klein et al. operated on 16 adult Swiss mountain sheep, in which a tibial fracture was induced (Klein et al. 2010). The fractures were stabilized with a static interlocking nail using an AO/ASIF 9.5 mm nail transfixed with 3.9 mm interlocking screws after using a 11 mm RIA. Non-reamed fractures were stabilized with a 7.5 mm nail ($n=8$ per group).

After the fracture and nailing procedure intravital staining with Procion red was performed. The effects of the nailing technique on cortical perfusion (Procion red) were evaluated in polymer embedded sections and cryosections. Cryosections stained with Sudan III were assessed with respect to cortical fat distribution. After irrigation and suction minute amounts of fat were observed in the cortex, whereas after non-reamed nailing the endosteal third of the cortical bone was penetrated with fat. Non-reamed nailing acutely showed better perfusion in the endosteal tenth and periosteal third of the cortical bone. After irrigation and suction reaming perfusion was preserved to a lesser degree. The authors concluded that the RIA is as efficient as its predecessors as the irrigation and suction significantly reduces fat intravasation, and thus the danger of system-wide damage.

40.6.2 Dogs

In 1988, Macdonald et al. (Skirving et al. 1987; Macdonald et al. 1988) reported a device for the reproducible creation of transverse fractures in canine tibiae utilizing a three-point bending technique. Fracture models of osteotomized long bones have been well characterized over the years in different large animal species. A number

of publications have described fracture models in dogs since the dog, beside pigs, is considered the most closely related model for research of human orthopaedic conditions. The effect of bending stiffness of external fixators on the early healing of transverse tibial osteotomies was described in a canine model by Gilbert (Gilbert et al. 1989). Tiedemann et al. assessed densitometric approaches to measure fracture healing in 6-mm tibial segmental defects and single-cut osteotomy defects in adult mongrel dogs (Tiedeman et al. 1990). Bilateral tibial transverse osteotomies were performed with a 2 mm gap by Markel et al. to quantify local material properties of fracture callus during gap healing (Markel et al. 1990). To compare the dosage-dependent efficacy of rhBMP-2 on tibial osteotomy healing, adult female dogs underwent right mid-shaft osteotomies with a 1 mm gap. The operated bones were stabilized using external fixators (Faria et al. 2007). In a similar study by Edwards, bilateral tibial osteotomies were performed to evaluate the capacity of a single percutaneous injection of rhBMP-2 delivered in a rapidly resorbable calcium phosphate paste (alpha-BSM) to accelerate bone healing (Edwards et al. 2004). The effect of shock wave therapy on acute tibial fractures was studied by Wang et al. in adult dogs after creation of bilateral tibial osteotomies with a 3 mm defined fracture gap (Wang et al. 2001). Similar models were also described by Hupel to compare the effects of unreamed and reamed nail insertion (Hupel et al. 2001), Jain et al. (1999) to investigate whether or not the limited contact design of the low-contact dynamic compression plate (LC-DCP) provides advantages over the dynamic compression plate (DCP) in the context of cortical bone blood flow, biomechanical properties, and remodelling of bone in segmental tibial fractures and Nakamura (Nakamura et al. 1998) to evaluate effects of recombinant human basic fibroblast growth factor (bFGF) on fracture healing in beagle dogs.

40.6.3 Pigs

Pigs are reported as the subject of choice in a variety of studies of bone remodelling, including osteonecrosis of the femoral head, cartilage defects, fractures, and studies evaluating new implant designs (Buser et al. 1991; Sun et al. 1999). The influence of systemic growth hormone application to stimulate bone metabolism and the underlying cellular mechanisms of fracture healing were analysed by Bail et al. (2002). A standardized osteotomy of 1 cm length was performed in the right tibia of Yucatan micropigs using an oscillating saw. The defect was stabilized with a low contact dynamic compression plate (LC-DCP) and the animals were divided into two groups. Animals in the treatment group received a single daily subcutaneously injection of 100 μg of recombinant species-specific growth hormone per kg body weight over 42 days, while the control group received sodium chloride as a placebo. The quantitative computer tomography measurement showed a significant higher bone mineral content in the growth hormone group than in the placebo group. The bone mineral density was comparable in both groups, whereas the torsional stiffness and the torsional failure load were higher in the growth hormone group compared to the control group. Hill and Watkins developed a model of ballistic wounds in the

proximal tibia of pigs (Hill and Watkins 2001). They could show that osteomyelitis can be prevented by the administration of systemic antibiotics, commencing at up to 6 h after surgery.

40.7 Tibial Segmental Defect Models

40.7.1 Sheep and Goats

In order to ascertain whether newly developed bone graft substitutes or tissue engineered constructs (TEC) comply with the requirements of biocompatibility, mechanical stability and safety, the materials must be subject to rigorous testing both *in vitro* and *in vivo*. To extrapolate results from *in vitro* studies to *in vivo* patient situations however is often difficult. Therefore, the application and systematic evaluation of new concepts in animal models is often an essential step in the process of assessing newly developed bone grafts prior to clinical use in humans. To simulate human *in vivo* conditions as closely as possible, a variety of large critical sized tibial defect models – mainly in sheep – have been developed over the past decade in order to investigate the influence of different types of bone grafts on bone repair and regeneration (Fig. 40.3). Critical sized segmental defects in long bones are usually defined by multiplying the shaft diameter by 2.0–2.5 (Lindsey et al. 2006; Gugala et al. 2007). Interestingly, the method of producing the gap may influence the outcome of those studies. Kuttenberger et al. could show that by using a CO_2-laser, the osteotomy ends were not as impaired in structure as when using an oscillating saw (Kuttenberger et al. 2008).

To evaluate the effects of different bioceramics on bone regeneration during repair of segmental bone defects Gao et al. (1997) implanted biocoral and tricalcium phosphate cylinders (TCP) in sheep tibial defects of 16 mm length. The defects were stabilized medially using two overlapping contoured auto-compression plates of 4 mm thickness (eight and six holes) and cortical screws. When compared to TCP, with the biocoral implants, a significant increase in external callus and density was seen after 3 weeks and an increase of torque capacity, maximal angle of deformation and energy absorption could be measured after 12 weeks while microscopically osseointegration appeared better. However, in his study, Gao used both male and female animals with a relatively large variation in body weight. Both factors, gender and body weight are known to have an influence on bone regeneration due to effects both on the biomechanical environment and hormonal feedback control mechanisms. Hence, variations in sex and body weight should be avoided. The defect fixation method used in this study can most likely be interpreted as a means to countervail bending forces on the implant after earlier failures. However, defect fixation by overlapping plates is not necessarily *lege artis* and has never been introduced and applied clinically to our knowledge. Therefore, a thicker and hence, stiffer plate should be chosen instead.

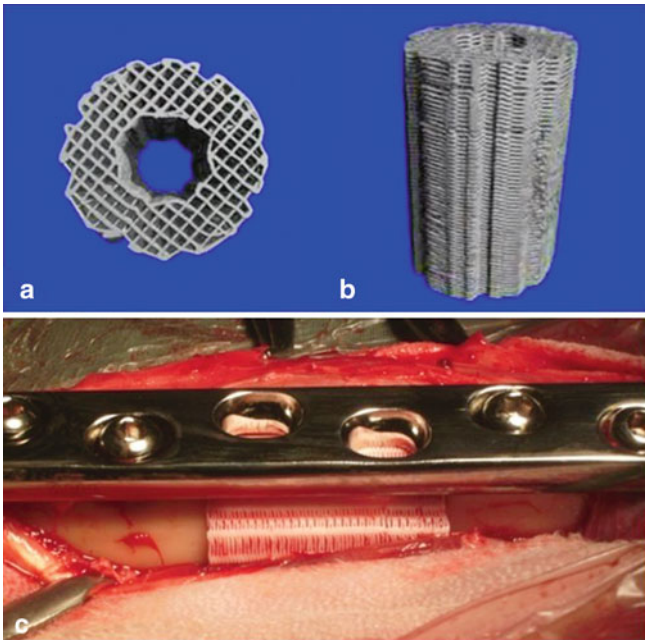


Fig. 40.3 MicroCT images of a medical grade ε-polycaprolactone β-tricalciumphosphate scaffold fabricated via fused deposition modelling (**a**, **b**). The scaffold has an outer diameter of 20 mm and a height of 30 mm. This scaffold type has been used to reconstruct a 30 mm critical sized defect in an ovine tibia (**c**)

Den Boer et al. reported a new segmental bone defect model where a 30 mm segmental defect was inflicted on sheep tibiae and stabilized by an interlocking intramedullary nail (custom made AO unreamed humeral nail). X-ray absorptiometry was applied to quantify healing (den Boer et al. 1999). Groups of this pilot study included untreated controls and autograft. After 12 weeks, despite higher bone mineral density in the autograft group, no significant difference in torsional strength and stiffness could be revealed. Since 33% of the control animals showed sufficient bridging of the defect, it needs to be questioned if the authors succeeded in establishing a reliable non-union model. Removal of the periosteum or a larger defect site might have been beneficial. In a subsequent study, the authors described the fabrication of biosynthetic bone grafts and their application in the very same animal model (den Boer et al. 2003). The five treatment groups included empty controls, autografts, hydroxyapatite alone, hydroxyapatite combined with rhOP-1, and hydroxyapatite with autologous bone marrow. At 12 weeks, healing of the defect was evaluated radiographically, biomechanically and histologically and revealed that torsional strength and stiffness were two fold higher for animals treated with autograft and hydroxyapatite plus rhOP-1 or bone marrow. Since healing was only evaluated after 12 weeks, no conclusions could be drawn regarding the process of healing. The mean values of both combination groups were comparable to those

of autografts. A higher number of defect unions was described when hydroxyapatite plus rhOP-1 was applied rather than hydroxyapatite alone. Analysing this study, it has to be taken into account that animals treated with hydroxyapatite and bone marrow were of a different breed with a higher average body weight. Animals were held at a different holding facility and accustomed to unequal forage all of which could possibly influence study outcomes.

Bone healing in critical sized segmental diaphyseal defects in sheep tibiae was also investigated by Gugala et al. (Gugala and Gogolewski 1999, 2002). Defects were bridged with a single porous tubular membrane or with anatomically shaped porous double tube-in-tube membranes. Membranes with different pore structures were applied alone and/or in combination with autologous bone graft. The diaphyseal defects were 40 mm in length and stabilized with a bilateral AO external fixator. Operated animals were 6–7 years of age. Of the six treatment groups however, only in groups where the defect was filled with autogenous cancellous bone graft and covered with a single perforated membrane or where the bone graft was administered in a space between a perforated internal and external membrane, could defect healing be observed. The authors partly contribute the healing effect to their membrane system, however a control group, where autologous bone graft is administered without any membrane was not described. It could also be criticized that post surgery animals were suspended in slings over the entire experimental period preventing the animals from sitting and therefore getting up, thus not reflecting the normal physiological load bearing conditions.

Wefer et al. (2000) conducted a study to develop and test a scoring system based on real-time ultrasonography to predict the healing of a bone defect filled with a porous hydroxyapatite bone graft substitute or cancellous bone graft from the iliac crest and stabilized by anterolateral plate osteosynthesis. After sacrifice tibiae were tested in torsion to failure. The results were correlated with radiographic and ultrasound scores obtained. Sheep with ceramic implants that developed non-unions showed a significantly lower score than sheep with sufficient implant integration. A significant correlation between these scores and the biomechanical results was found. However, although the authors describe their 20 mm defect as a critical sized model, no control group was included for comparison. Hence, the critical nature of the defect in this study can be questioned.

The effects of new resorbable calcium phosphate particles and paste forms, which harden in situ after application, on bone healing were investigated by Bloemers et al. (2003). They used a 30 mm segmental tibial defect fixed by a custom made AO unreamed interlocking titanium tibial nail. Twelve weeks after defect reconstruction, radiological, biomechanical, and histological examinations were performed. Radiographically, the resorbable paste group performed better than all other groups. Biomechanical tests revealed a significantly higher torsional stiffness for the resorbable calcium-phosphate paste group in comparison with autologous bone. The study indicated that new calcium phosphate based materials might be a potential alternative for autologous bone grafts in humans. As with several other studies critically reviewed in this article, animals of a minimum age of 2 years with a significant variation in body weight were used in this study. As mentioned before,

it must be considered that secondary osteonal bone remodelling in sheep does not occur until an age of 7–9 years. Therefore, it might be difficult to extrapolate results from this study for applications in adult human patients as human bone primarily undergoes secondary osteonal bone remodelling. Insulin-like growth factor I (IGF-1) exerts an important role during skeletal growth and bone formation. Therefore, its localized delivery appears attractive for the treatment of bone defects. To prolong IGF-1 delivery, Meinel et al. entrapped the protein into biodegradable poly(lactide-co-glycolide) microspheres and evaluated the potential of this delivery system for new bone formation in a non-critical 10 mm segmental tibia defect (Meinel et al. 2003). The defect was stabilized using a 3.5 mm 11 hole DCP. Administration of 100 μg of IGF-1 in the microspheres resulted in bridging of the segmental defect within 8 weeks. To avoid excessive load on the operated limbs and fracturing of the freshly operated tibial defects, the animals were accommodated in a suspension system for a period of 4 weeks postoperatively. When interpreting data published in this study, it must be taken into account that the close position of the screws to the defect proximally and distally, and the obvious fact that the screws at the defect site had not been inserted at a defined angle might have influenced and biased the outcomes.

In a 48 mm tibial defect model in sheep ceramic implants of 100% synthetic calcium phosphate multiphase biomaterial were evaluated (Mastrogiacomo et al. 2006). The defect was stabilized with a 4.5 mm neutralizing plate. Although not reported by the authors, one can observe bent plates and axial deviations in presented x-ray and CT images, hence, from a clinical point of view, it must be concluded that the chosen fixation in that model seemed not to be sufficient. The x-ray series of the 2-year animal suggests that the internal fixation device had been explanted 12–14 weeks post surgery, a fact not described and explained by the authors. Assuming recovery and bone regeneration without any complications, in human patients, internal fixation devices would usually not be removed until 12–18 months post implantation. Good integration between the ceramic implants and the adjoining proximal and distal bone ends was observed. A progressive increase in new bone formation was seen over time, along with progressive resorption of the ceramic scaffold. Based on x-ray analysis, at the 1-year time point, approximately 10–20% of the initial scaffold substance was still present, and after 2 years it was almost completely resorbed. The authors state that approximately 10–20% of the periosteum was deliberately left in situ as a source of osteogenic cells. However, one might conclude that this procedure appears to be rather difficult to standardize in order to develop a reproducible model.

Another study using an ovine segmental defect model investigated the influence of rhTGFβ-3 on mechanical and radiological parameters of a healing bone defect (Maissen et al. 2006). In 4–5-year-old sheep, an 18 mm long osteoperiosteal defect in the tibia fixed with a unilateral external fixator was treated by rhTGFβ-3 delivered by a poly(L/DL-lactide) carrier, with the carrier only, with autologous cancellous bone graft, or remained untreated. Weekly *in vivo* stiffness measurements and radiological assessments were undertaken as well as quantitative computed tomographic assessments of bone mineral density in 4-week intervals. The follow up of

the experiment was 12 weeks under partial weight bearing since animals were kept in a support system to prevent critical loads on the fixator and its interface to bone thus not reflecting physiological loading conditions. The 18 mm defect size described as spontaneously non-healing, might not have been sufficient to establish a non-union model in a fully weight bearing biomechanical environment. In the bone graft group, a significantly higher increase in stiffness was observed than in the PLA/rhTGFβ-3 group and a significantly higher increase than in the PLA-only group. The radiographic as well as the computer tomographic evaluation yielded significant differences between the groups, indicating the bone graft treatment performed better than the PLA/rhTGFβ-3 and the PLA-only treatment.

Sarkar et al. assessed the effect of platelet rich plasma (PRP) on new bone formation in a 25 mm diaphyseal tibial defect in sheep (Sarkar et al. 2006). The defect was stabilized with a custom-made intramedullary nail (stainless steel, diameter proximal 12 mm, distal 10 mm) with two locking screws each proximally and distally. To reduce stress at the screw/bone interface, a custom-made stainless steel plate was additionally applied medially representing an unconventional fixation method not found in the clinic. However, no reasoning for the additional medial plating was provided in the publication by the authors. Defects were treated with autogenous PRP in a collagen carrier or with collagen alone. A control group to establish the critical nature of the defect was not included and has therefore to be questioned. After 12 weeks, the explanted bone specimens were quantitatively assessed by x-ray, computed tomography (CT), biomechanical testing and histological evaluation. Bone volume, mineral density, mechanical rigidity and histology of the newly formed bone in the defect did not differ significantly between the PRP treated and the control group, and no effect of PRP upon bone formation was observed.

In 2007 Tyllianakis et al. (2007) determined the size of a bone defect that can be restored with one-stage lengthening over a reamed intramedullary nail in sheep tibiae. Sixteen adult female sheep were divided into four main groups: a simple osteotomy group (group I) and three segmental defect groups (10, 20, and 30 mm gaps, groups II-IV). One intact left tibia from each group was also used as the non-osteotomized intact control group (group V). In all cases, the osteotomy was fixed with an interlocked Universal Humeral Nail (UHN-Protek-Synthes). Healing of the osteotomies was evaluated after 16 weeks by biomechanical testing. The examined parameters were torsional stiffness, shear stress, and angle of torsion at the time of fracture. The regenerated bone obvious in x-rays in the groups with 10 and 20 mm gaps had considerable mechanical properties. Torsional stiffness in these two groups was nearly equal and its value was about 60% of the stiffness of the simple osteotomy group. Gradually decreasing stiffness was observed as the osteotomy gap increased. No significant differences were found between the angles of torsion at fracture for the various osteotomies or the intact bone. These results showed that the group with the 10 cm gap had 65% of the shear stress at failure compared to the simple osteotomy group.

Teixeira et al. treated tibial segmental defects of 35 mm size in both male and female sheep aged 4–5 months. Considering the age of the animals and the preservation of the periosteum, the critical size of this defect can be questioned and

results cannot necessarily be extrapolated to adult humans, as described correctly by the authors. An empty control group was not included in the experiment. The bone defects in the diaphysis of the right hind limb were stabilized with a titanium bone plate (103 mm in length, 2 mm thickness, and 10 mm width) combined with a titanium cage. As reported by the authors, plate bending occurred in 42% of the animals and was partly attributed to the connection of the titanium cage to the plate. However, it appears that the bending of the plate was rather a result of insufficient thickness of the fixation device. The titanium cages were either filled with autologous cortical bone graft or with a composite biomaterial consistent of inorganic bovine bone, demineralised bovine bone, a pool of bovine bone morphogenetic proteins bound to absorbable ultra-thin powdered hydroxyapatite and bone-derived denaturized collagen. Bone defect healing was assessed clinically, radiographically and histologically. Titanium cages might keep implanted scaffolds and biomaterials in place initially and biomechanically support defect fixation, however, it must be taken into consideration that – since titanium is not resorbable – the cages might hinder complete bone remodelling in the long run.

Radiographic examination showed initial formation of periosteal callus in both groups at osteotomy sites, over the plate or cage 15 days postoperatively. At 60 and 90 days callus remodelling occurred. Histological and morphometric analysis 90 days post surgery showed that the quantity of implanted materials still present were similar for both groups while the quantity of newly formed bone was less ($p = 0.0048$) in the cortical bone graft group occupying $51 \pm 3.46\%$ and $62 \pm 6.26\%$ of the cage space, respectively (Teixeira et al. 2007).

Recently, Liu et al. reported on the use of highly porous beta-TCP scaffolds to repair goat tibial defects (Liu et al. 2008). In this study, 15 goats were randomly assigned to one of three groups, and a 26 mm long defect at the middle part of the right tibia in each goat was created and stabilized using a circular external fixator. In Group A, a porous beta-TCP ceramic cylinder that had been loaded with osteogenically induced autologous bone marrow stromal cells was implanted in the defect of each animal. In Group B, the same beta-TCP ceramic cylinder without any cells was placed in the defect. In Group C, the defect was left untreated. In Group A, bony union could be observed by gross view, x-ray and microcomputed tomography (μCT) detection, and histological observation at 32 weeks post-implantation. The implanted beta-TCP scaffolds were almost completely replaced by host bone. Bone mineral density in the repaired area of Group A was significantly higher than in Group B, in which scant new bone was formed in each defect and the beta-TCP hadn't been completely resorbed after 32 weeks. Moreover, the tissue-engineered bone of Group A had similar biomechanical properties as the contralateral tibia in terms of bending strength and Young's modulus. In Group C, little or no new bone was formed and non-union occurred, demonstrating the critical nature of the defect.

Using 32 male adult goats (aged 3, average weight 50 kg), Liu et al. investigated the effect of fiber-reinforced scaffolds consisting of nano-hydroxyapatite/collagen/poly (L-lactic acid) (PLLA)/chitin fibers (nHACP/CF) (Liu et al. 2010). Defects of 25 mm length were created and stabilized with a four-hole steel plate. The scaffolds were transplanted alone or in combination with bone marrow derived mesenchymal

cells and compared to autograft from the iliac crest. Animals were kept for 4 and 8 weeks (n = 4), respectively. Quantitative histological assessment showed the highest amount of bone with autograft followed by the scaffold with cells and the scaffold alone. After 8 weeks four out of four defects were bridged after autograft and scaffold/cell transplantation. Three point bending showed a load to failure of the repaired tibiae in the scaffold/cell group of 1,396.6 ± 138.2 N, and of 1,402.3 ± 99.6 N in the autograft group.

To investigate the effect of chondroitine sulphate on bone remodelling and regeneration, Schneiders et al. (2008) created a 30 mm tibial mid-diaphyseal defect site and reconstructed it using hydroxyapatite/collagen cement cylinders. Defect stabilization was achieved by insertion of a universal tibial nail (UTN, Synthes, Bochum). However, to place the scaffold into the defect, the authors had to use a second operative aditus mid-diaphyseally. The published data suggest problems with defect fixation in form of implant failures. Moreover, signs of locking bolt loosening, poor contact between bone and nail, and the proximal nail end extending into the articular space were evident, facts not reported by the authors. In addition, it can be supposed that either the insertion of the nail or undesired movement of the loosened nail has caused damage to the cylindrical biomaterials at testing. When interpreting the acquired data, it also has to be taken into account that obviously no fabrication method has been described to reliably reproduce implants of corresponding geometrical shape.

Rozen et al. investigated whether blood-derived endothelial progenitor cells promote bone regeneration once transplanted into an ovine, critical sized, tibial defect (Rozen et al. 2009). Cells were isolated and expanded *in vitro*. 2×10^7 cells were resuspended in 0.2 ml saline were transplanted 2 weeks after a 3.2 cm defect had been created (n = 7). Defect fixation was achieved by a 4.5 mm stainless steel plate with four screws each proximally and distally. In the control group (n = 8) only 0.2 ml saline were injected. Defect bridging was observed in six out of seven animals in the experimental group. In the control group, five out of six defects analysed via μCT showed discontinuous (two animals) or minute bridging (three animals) as stated by the authors. No reference to the remaining three animals of the control group was found throughout the manuscript. Therefore, the critical nature of the defect has to be questioned. Not resecting the periosteum and screw loosening as clearly evident in the published x-ray images might have contributed to defect bridging in the control group.

The regenerative capacity of xenogenic human and autologous ovine mesenchymal progenitor cells was assessed by Niemeyer et al. in an ovine critical-size defect model (Niemeyer et al. 2009). Human and ovine MSC from bone marrow, were cultured on mineralized collagen and implanted into a 3.0 cm ovine tibial bone defect (n = 7). Unloaded mineralized collagen served as control. The 3 cm mid-diaphyseal defects were fixed with a seven-hole LC-LCP (Synthes) and a carbon fibre reinforced poly-ether-ketone plate (snakeplate, Isotec AG, Altstätten, Switzerland). Animals were kept in suspending slings for 8 weeks post surgery. Nevertheless, implant failure occurred in one animal requiring immediate euthanasia. Wound healing related problems were reported for another animal. Bone healing was

assessed up to 26 weeks. Presence of human cells after xenogenic transplantation was analysed using human-specific *in situ* hybridization. Radiology and histology demonstrated significantly better bone formation after transplantation of autologous ovine MSC on mineralized collagen compared to unloaded matrices and to the xenogenic treatment group. No local or systemic rejection reactions could be observed after transplantation of human MSC and although the presence of human MSC could be demonstrated.

In another study, the same group compared the osteogenic potential of bone marrow derived mesenchymal stem cells (BMSC) and adipose-tissue derived stem cells (ASC) and evaluated the influence of platelet rich plasma (PRP) on the osteogenic capacity of ASC using the same set up and evaluation methods (Niemeyer et al. 2010). Ovine BMSC (BMSC-group) and ASC (ASC-group) were seeded on mineralized collagen sponges and implanted into a critical size defect of the sheep tibia ($n=5$ each). In an additional group, platelet-rich plasma (PRP) was used in combination with ASC (PRP-group). Unloaded mineralized collagen (empty group) served as a control ($n=5$ each). Radiographic evaluation revealed a significantly higher amount of newly formed bone in the BMSC-group compared to the ASC-group at week ten ($p<0.05$). In contrast to ASC, PRP application led to significantly more bone when compared to the empty control group ($p<0.05$). These findings were confirmed by histological analysis.

Recently, Huang et al. reported on the design and fabrication of laminated scaffolds for the repair of bone defects (Huang et al. 2011). The scaffolds consisted of β-tricalcium phosphate (β-TCP) and poly (L-lactic acid) (PLLA) and were of cylindrical shape. Porosity and bending strength of the scaffolds were around 70% and 1.7 MPa, respectively. Caprine diaphyseal tibial defects (20 animals) of 3 cm length were created, stabilized with a stainless steel plate, and left untreated or filled with scaffolds loaded with allogeneic BMSCs. Compared to control, scaffold/cell transplantation increased the rate of bone formation after 12 weeks. As in many other studies, the information provided does not allow for reproduction of the results.

Wang et al. treated a number of 12 segmental goat defects of 30 mm length (six animals, bilateral defects) with porous β-TCP cylinders (porosity 70%; pore size 450 μm; diameter 16 mm; height 30 mm; Shanghai Bio-Lu Biomaterials Co., Ltd. Shanghai, China). The scaffolds had a compressive strength of 2-4 MPa. The scaffolds were seeded with bone marrow derived cells in a perfusion reactor or cultured under static conditions prior to implantation. Animals were kept for 24 weeks. One animal did not survive the experimental period. After 24 weeks, the regenerated defects were analyzed by histology and micro-CT. The results showed an increased amount of bone formation for the scaffold group cultured under dynamic conditions ($p<0.05$).

The efficacy of allogeneic mesenchymal precursor cells for the repair of an ovine tibial segmental defect was assessed recently (Field et al. 2011). Twenty-four, mature female sheep underwent surgery for the creation of a 3 cm tibial diaphyseal defect. In one group of 12 sheep a scaffold was used alone (MasterGraft Matrix, Medtronic), and in the second group the scaffold was seeded with allogenic MPC isolated from bone marrow aspirates. The defect was stabilised using a locking

intramedullary nail and allowed to heal over a 9-month-period. The MPC-treated group displayed a significantly greater level of callus formation and rate of bone apposition in the defect. The incorporation of allogeneic MPC to the synthetic void filler stimulated early repair of critical-size diaphyseal segmental defects.

Our own research group has described a non-critical tibial segmental defect model. The model was used to compare the regenerative potential of scaffolds with different material composition but similar mechanical properties to autologous bone graft from the iliac crest. Twelve Merino sheep (weight 42 ± 2 kg, age 7 years) were included in the study, in which a tibial segmental defect of 2 cm length was created. The defects were stabilized with a 4.5 mm limited contact locking compression plate (LC-LCP, Synthes). They were left untreated, filled with autologous cancellous bone graft from the iliac crest or medical grade polycaprolactone (mPCL-TCP) or poly(L-lactide-co-D,L-lactide) (PLDLLA)-TCP-PCL scaffolds. After 12 weeks, *in vivo* specimens were analyzed by X-ray imaging, torsion testing, micro-computed tomography and histology to assess amount, strength and structure of the newly formed bone. The highest amounts of bone neoformation with highest torsional moment values were observed in the autograft group and the lowest in the mPCL-TCP composite group. The study results suggested that scaffolds based on aliphatic polyesters and ceramics, which are considered biologically inactive materials, induce only limited new bone formation but could be an equivalent alternative to autologous bone when combined with a biologically active stimulus (Reichert et al. 2011)(Fig. 40.4).

We have also succeeded in establishing a critical-sized segmental defect model in sheep (Reichert et al. 2010). In a following study, such critical-sized 3 cm defects were stabilized with a dynamic compression plate (DCP, Synthes) and left untreated, reconstructed with autologous bone graft (ABG), mPCL-TCP or silk-HA (hydroxyapatite) scaffolds. Animals were held for 12 weeks. X-ray analysis, torsional testing and quantitative CT analysis were performed. Radiologic analysis confirmed the critical nature of the defects. Full defect bridging occurred in the autograft partial bridging in the mPCL-TCP group. Only little bone formation was observed with silk-HA scaffolds. Biomechanical testing revealed a higher torsional moment/stiffness ($p < 0.05$), CT analysis a significantly higher amount of bone formation for the ABG group when compared to the silk-HA group. No significant difference was determined between the ABG and mPCL-TCP group. It was concluded that the combination of mPCL-TCP with osteogenic cells or growth factors might represents an attractive means to further enhance bone formation (Reichert et al. 2012) (Fig. 40.5).

Continuing work pertains to the use of mesenchymal progenitor cells and/or recombinant growth factors to stimulate bone growth in defects of critical size.

40.7.2 Dogs

Tiedemann et al. developed a non-invasive method to assess fracture healing using densitometric methodology, tested in 6 mm tibial segmental defects in adult mongrel dogs (Tiedeman et al. 1990). The lowest measurable bone density in the

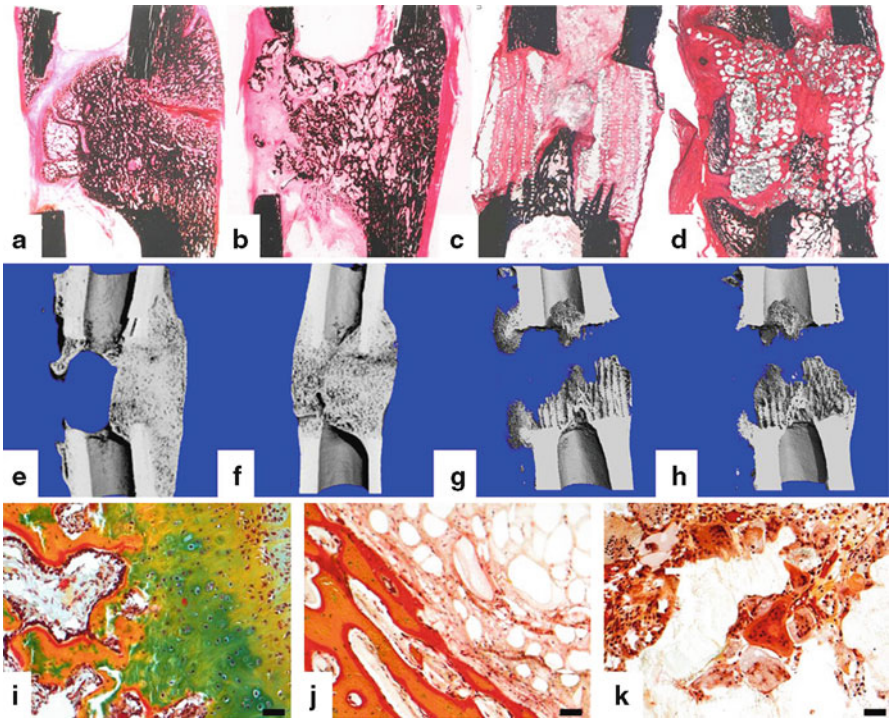


Fig. 40.4 Von Kossa/van Gieson staining on PMMA embedded specimens (**a**–**d**) showed extensive bone formation (*black*) and bridging in the autograft group (**b**, **f**), considerably less bone formation and nonunion in the empty control (**a**, **e**) and the scaffold groups (mPCL-TCP: **c**, **g**; PDLLA-TCP-PCL: **d**, **h**). Histology results correlated well with 3-D microCT reconstructions of the defects (**e**–**h**). Movat's pentachrome staining (**i**–**k**) suggested endochondral bone formation in all groups (**i**, cartilage, *green*) with subsequent osteoid formation (**j**). In the autograft group, first signs of bone remodelling were evidenced by osteoclasts and giant cells within the defect area (**k**)

defect was compared to bending rigidity of the involved extremity. They found a highly significant correlation between densitometric evaluation and bone rigidity. The changes in biomechanical characteristics during the healing process of experimental transversal tibial mid-shaft osteotomies in 21 beagle dogs were investigated by Hara et al. (2003). The hindleg was stabilized with an intramedullary pin and an external coaptation was applied with a hardening bandage for 4 weeks after surgery. Observation at 2, 4, 8, 16, and 32 weeks showed that the biomechanical characteristics of the healing bone could not recover sufficiently even after the passage of the healing period, which has been empirically proposed from clinical findings. In a study by Kokubo et al. the long-term stability of bone tissues induced by rhBMP-2 and poly(L-lactide-co-glycolide) co-polymer-coated gelatin sponge (PGS) was examined (Kokubo et al. 2004). A 2.5 cm long unilateral bone defect in the left tibia was created in 16 male beagle dogs and stabilized with a

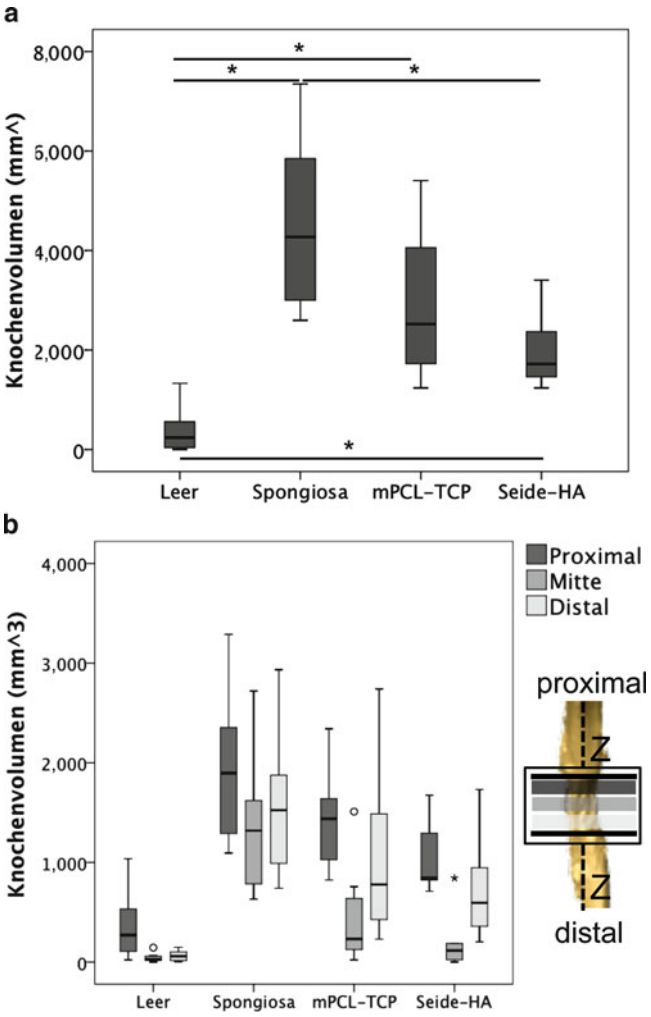


Fig. 40.5 Quantitative analysis of CT scans 12 weeks after surgery. The total volume of newly formed bone within the defect (**a**) and the bone volume distribution to the proximal, middle and distal defect third (**b**) was calculated. The *box plots* indicate the median, first and third quartile as well as maximum as minimum values. *Bars* and *asterisks* indicate statistical significance

metal plate. The metal plates of the rhBMP-2 treated limbs were removed after 16 weeks and biomechanical testing and histological analyses were performed after 32, 52, and 104 weeks. The hindlimbs were supported with a glass fiber cast for 4 weeks after plate removal. All defects treated with rhBMP-2 achieved radiographic bony union after 8 weeks, whereas the defects treated with PGS alone resulted in non-unions at 16 weeks. No statistical significances were detected in all parameters between regenerated and intact tibiae at 104 weeks. To compare

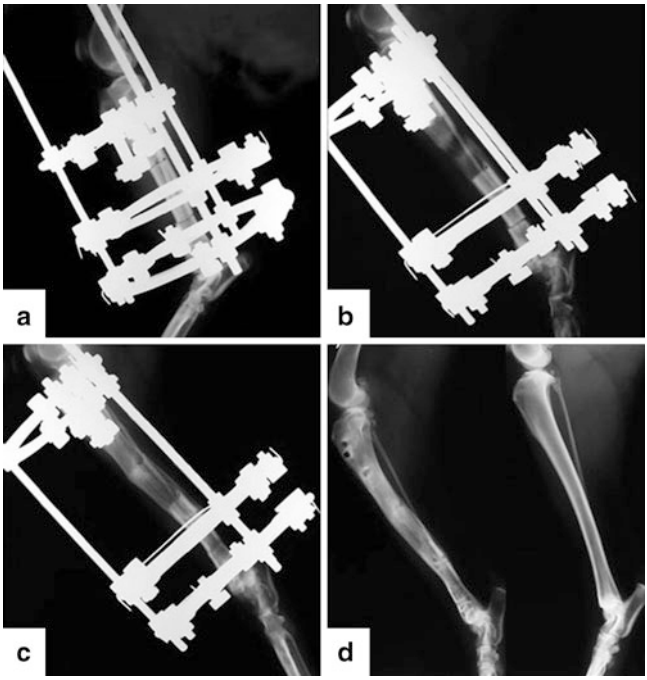


Fig. 40.6 Radiography of a right dog tibia submitted to acute bone shortening achieved by resection of 30% of its length. Tibia shortening was followed by gradual distraction at a rate of 1 mm/day until the excised fragment length was regained. The fixator was then locked to support the newly formed bone for another 14 weeks. The images show well-positioned fragments post surgery (**a**). At 6 weeks of bone distraction longitudinal radiodense columns in the direction of the distraction from both ends of the original bone could be observed (**b**) resulting in bone regeneration within the distraction gap at week 14 (**c**) of the neutral fixation period. The lateral radiography (**d**) demonstrates the final appearance 4 weeks after frame removal

their treatment concept with the established Ilizarov technique, Rahal et al. used a segmental tibia defect model for the treatment of acute bone shortening followed by gradual lengthening with circular external fixator (Rahal et al. 2005) (Fig. 40.6). In seven female dogs, 30% (average 4.75 cm) of the tibia and fibula including the periosteum were removed and acute bone shortening of the proximal and distal segments was performed. After 6 days, bone distraction was started at a rate of 1 mm/day until a distraction equal in length to the removed bone segment was achieved. After lengthening, the fixator was left in place for 14 weeks for consolidation of regenerated bone. Functional results were considered excellent in two, good in three and fair in the other two dogs. Radiographic images showed bone regeneration within the distraction gap after 14 weeks. Itoh et al. used a hydroxyapatite/type I collagen (HAp/Col) composite loaded with rhBMP-2 for the treatment of 2 cm long segmental bone defects in the tibia of six male beagle dogs (Itoh et al. 2002). The implants were fixed with an Ilizarov external fixator. The change of bone mineral density as well as radiological and histological

findings suggest that the implants were able to induce bone remodelling units and are a suitable carrier for rhBMP-2 due to the stimulation of early callus and new bone formation.

40.7.3 Pig

Windhagen et al. developed a new method for quantitative evaluation of *in vivo* bone regeneration in distraction osteogenesis in micropigs (Windhagen et al. 2000). To measure the *in vivo* stiffness they used a newly developed bone-healing meter. After euthanasia of the pigs, they quantified the maximum torsional moment data for the regenerated bones. When analysing the data, a highly significant regression between *in vivo* stiffness and maximal torsional moment was found. The authors concluded that their method may be a reliable tool for future quantitative monitoring of healing progress in patients.

The effect of homologous recombinant porcine growth hormone (r-pGH) on secondary fracture healing was investigated in a diaphyseal defect of the tibia in 24 Yucatan micropigs by Raschke et al. (2001). A 1 cm defect of the tibia was surgically created and stabilized with an AO 3.5 mm dynamic compression plate (DCP). The treatment group received 100 μg of r-pGH per kilogram of body weight subcutaneously once per day. Quantitative computed tomography (qCT) and biomechanical analysis was performd after 6 weeks. qCT measurements revealed a significant increase in the bone mineral content of the defect zone in the treatment group compared with controls, whereas the bone mineral density values were similar in both groups. Torsional failure load was higher in the treatment group than in the control group. The results showed that a daily application of recombinant GH stimulates secondary fracture healing, resulting in increased mechanical strength and stiffness of the callus.

Pek et al. created a porous bioresorbable nano-composite scaffold for the treatment of segmental bone defects (Pek et al. 2008). A 2×1 cm tibial bone defect was created in 10 Yorkshire-Landrace pigs, treated with the scaffolds and stabilized with a 7-hole limited contact dynamic compression plate (LC-DCP). After 3 and 6 month histological examinations, dual energy X-ray absorptiometry (DEXA), bone mineral density (BMD) and computed tomography (CT) scans were performed. The nano-composite scaffold demonstrated excellent bioactivity for promoting cell attachment and proliferation, it was osteoinductive and successfully healed a critical-sized defect in the pig tibia.

40.8 Metatarsal Fracture Models

Only few groups have applied metatarsal fracture models to study bone regeneration. A PubMed search including publications of the last 15 years listed publications from one research group only. Prof. Lutz Claes (University of Ulm, Germany) and

co-workers have published a number of original research articles investigating the effect of dynamisation on gap healing of diaphyseal fractures under external fixation (Claes et al. 1995), the effects of mechanical factors on the fracture healing process (Claes et al. 1998), local tissue properties in bone healing (Augat et al. 1998), and the effect of mechanical stability on local vascularisation, tissue differentiation in callus healing (Claes et al. 2002), and cortical remodeling after osteotomy (Augat and Claes 2008).

40.9 Metatarsal Segmental Defect Models

In 2000 Nature Biotechnology published a study where 25 mm metatarsal defects fixed with a 3.5 mm narrow DCP were reconstructed with coral implants with and without MSC or freshly aspirated bone marrow (Petite et al. 2000). While hardly any bone formation was observed in the empty control group, coral scaffolds induced new bone formation confirming the osteoconductivity of the material. The combination of the scaffold with fresh bone marrow aspirates was not able to provide a sufficient bone formation stimulating signal leading to fibrous non-union in all defects. When combined with passage 2 MSCs (10^7 cells/scaffold) however, three animals showed clinical union after 16 weeks (n = 7).

Over the years, the same research group has published several studies relying on the same model (Viateau et al. 2004, 2006, 2007). In eleven 2-year-old Pré-Alpes Sheep mid-diaphyseal metatarsal bone defects (25 mm) were stabilized with a dynamic compression plate over a polymethylmethacrylate (PMMA) cement spacer, and by external coaptation. The PMMA spacer was removed after 6 weeks by incising the encapsulating membrane. The defect remained unfilled (n = 5) or was filled with morselized autologous corticocancellous graft (Group 2; n = 6), the membrane closed, and external coaptation applied for 6 months. Radiographic, computed tomographic, and histologic examinations 6 months after the second surgery revealed non-union in ungrafted defects whereas grafted defects showed bone healing. The induced membrane contained blood vessels, cbfa-1 positive cells, and very few macrophages entrapped in a collagenous tissue. It was concluded that the PMMA-induced membrane may help confine bone morphogenetic proteins, skeletal stem cells, or other agents to the defect cavity where they could possibly enhance bone formation. In a recent study, MSC seeded coral granules were transplanted into these defects with preformed membranes. Radiographic, histological, and computed tomographic tests performed after 6 months showed that the osteogenic abilities of the engineered construct and autograft were significantly greater than those of coral scaffold alone. No significant differences were found between the amount of newly formed bone in defects filled with coral/MSCs and those filled with autograft.

Claes et al. have examined the effect of the stiffness of the axial fixator on reducing the time of callus maturation (Claes et al. 2000). Therefore, a mid-diaphyseal defect of 15 mm was created in the metatarsal bone in sheep and stabilised with a ring fixator. After 4 days a bony segment was transported over a period of 16 days at a

rate of 1 mm/day. After 64 days, animals were divided into four groups, three with axial interfragmentary movement (IFM) of 0.5, 1.2 and 3.0 mm, respectively, and a control group. The 3.0 mm IFM group showed the smallest values for bone density ($p=0.001$) and area of callus and the largest IFM after 12 weeks. In this group also typical clinical signs of hypertrophic non-union were evident. The most rapid stiffening of the callus was seen in the 0.5 mm group, which had the smallest IFM ($p=0.04$) after 12 weeks along with radiological signs of defect bridging. The results indicated that suitable dynamic axial stimulation can enhance maturation of distraction callus when the initial amplitude is small, but that a large IFM can lead to delayed union. In the same model of distraction osteogenesis, they determined whether low-intensity ultrasound can be used to enhance callus maturation (Claes et al. 2005). Eighteen sheep were operated on and divided into two groups. One group was treated with low-intensity ultrasound for 20 min/day, whereas the other group served as an untreated control group. Biomechanical tests after removal of the metatarsals showed significantly higher axial compression stiffness and significantly higher indentation stiffness of callus tissue in the healing zone in the group treated with ultrasound. Also, histologic analysis of the cortical defect zone showed significantly more callus formation and more active zones of endochondral ossification in the group treated with ultrasound. The authors therefore concluded that stimulation of callus maturation by ultrasound is possible, similar to stimulation of fresh fracture healing, and may be used to shorten clinical treatment times.

As mentioned earlier, limited integration and remodeling can cause graft failure. Therefore, Di Bella et al. investigated whether mesenchymal stem cells (MSCs) and osteogenic protein-1 (OP-1) can improve allograft integration (Di Bella et al. 2010). In 20 alpine sheep (age 3–4 years, weight 60–70 kg), a 3 cm segmental bone defect was created in the mid-diaphysis of the metatarsal. Defects were stabilized using a seven-hole dynamic compression plate (titanium). They were augmented with an allograft alone (control group), or with MSCs, OP-1, or MSCs and OP-1. Radiographic anlysis showed accelerated graft integration with MSC and OP-1. Histology also demonstrated a significantly higher amount of bone within the graft and a higher vascularization for this group. Consequently, the authors concluded that MSC and OP-1 promote graft integration.

40.10 Summary

The reconstruction of complicated fractures and large segmental bone defects remains a significant clinical problem. Large bone defects may occur as a result of extensive bone loss resulting from pathological events such as trauma, inflammation, and tumour resection. Present therapeutic approaches include the application of bone graft transplants (autologous, allogenic, xenografts), fixation devices consisting of different synthetic and natural biomaterials, and segmental bone transport. However, to date, no existing therapy has been fully satisfactory. A number of research groups therefore work on the development of new bone grafting materials,

carriers, growth factors, and tissue engineered constructs for bone regeneration. These groups are interested in evaluating their concepts in reproducible large animal models. To optimize cell-scaffold combinations and the application of locally or systemically active stimuli remains a complex process. It is characterized by a highly interdependent set of variables with a large range of possible variations. Consequently, the evaluation of new developments in the field of bone tissue engineering must base on clinical experience, knowledge of basic biological principles, medical necessity, and commercial practicality. The area of bone tissue engineering relies on animal models to evaluate both experimental and clinical hypotheses. To overcome current limitations associated with bone tissue engineering, researchers must rely on the functional assessment of biological and biomechanical parameters of generated constructs. For comparison of different studies and their outcomes, the standardization of animal models, fixation devices, surgical procedures and methods of taking measurements is essential to accumulate a reliable data pool guiding further directions to orthopaedic and tissue engineering developments.

References

Aerssens J, Boonen S, Lowet G et al (1998) Interspecies differences in bone composition, density, and quality: potential implications for in vivo bone research. Endocrinology 139:663–670

Anderson ML, Dhert WJ, de Bruijn JD et al (1999) Critical size defect in the goat's os ilium: a model to evaluate bone grafts and substitutes. Clin Orthop Relat Res 364:231–239

Augat P, Claes L (2008) Increased cortical remodeling after osteotomy causes posttraumatic osteopenia. Bone 43:539–543

Augat P, Merk J, Genant HK et al (1997) Quantitative assessment of experimental fracture repair by peripheral computed tomography. Calcif Tissue Int 60:194–199

Augat P, Margevicius K, Simon J et al (1998) Local tissue properties in bone healing: influence of size and stability of the osteotomy gap. J Orthop Res 16:475–481

Augat P, Burger J, Schorlemmer S et al (2003) Shear movement at the fracture site delays healing in a diaphyseal fracture model. J Orthop Res 21:1011–1017

Bail HJ, Kolbeck S, Krummrey G et al (2002) Systemic application of growth hormone for enhancement of secondary and intramembranous fracture healing. Horm Res 58(suppl 3):39–42

Bloebaum RD, Ota DT, Skedros JG et al (1993) Comparison of human and canine external femoral morphologies in the context of total hip replacement. J Biomed Mater Res 27:1149–1159

Bloemers FW, Blokhuis TJ, Patka P et al (2003) Autologous bone versus calcium-phosphate ceramics in treatment of experimental bone defects. J Biomed Mater Res B Appl Biomater 66:526–531

Blokhuis TJ, Wippermann BW, den Boer FC et al (2000) Resorbable calcium phosphate particles as a carrier material for bone marrow in an ovine segmental defect. J Biomed Mater Res 51:369–375

Blokhuis TJ, den Boer FC, Bramer JA et al (2001) Biomechanical and histological aspects of fracture healing, stimulated with osteogenic protein-1. Biomaterials 22:725–730

Brodke D, Pedrozo HA, Kapur TA et al (2006) Bone grafts prepared with selective cell retention technology heal canine segmental defects as effectively as autograft. J Orthop Res 24:857–866

Bucholz RW, Carlton A, Holmes R (1989) Interporous hydroxyapatite as a bone graft substitute in tibial plateau fractures. Clin Orthop Relat Res 240:53–62

Bullens PH, Hannink G, Verdonschot N et al (2010) No effect of dynamic loading on bone graft healing in femoral segmental defect reconstructions in the goat. Injury 41:1284–1291

Buser D, Schenk RK, Steinemann S et al (1991) Influence of surface characteristics on bone integration of titanium implants: a histomorphometric study in miniature pigs. J Biomed Mater Res 25:889–902

Cacchioli A, Spaggiari B, Ravanetti F et al (2006) The critical sized bone defect: morphological study of bone healing. Ann Fac Med Vet di Parma 26:97–110

Chapman MW, Bucholz R, Cornell C (1997) Treatment of acute fractures with a collagen-calcium phosphate graft material: a randomized clinical trial. J Bone Joint Surg Am 79:495–502

Cierny G III, Zorn KE (1994) Segmental tibial defects: comparing conventional and Ilizarov methodologies. Clin Orthop Relat Res 301:118–123

Claes LE, Wilke HJ, Augat P et al (1995) Effect of dynamization on gap healing of diaphyseal fractures under external fixation. Clin Biomech (Bristol, Avon) 10:227–234

Claes LE, Heigele CA, Neidlinger-Wilke C et al (1998) Effects of mechanical factors on the fracture healing process. Clin Orthop Relat Res 355:S132–S147

Claes L, Laule J, Wenger K et al (2000) The influence of stiffness of the fixator on maturation of callus after segmental transport. J Bone Joint Surg Br 82:142–148

Claes L, Eckert-Hubner K, Augat P (2002) The effect of mechanical stability on local vascularization and tissue differentiation in callus healing. J Orthop Res 20:1099–1105

Claes L, Ruter A, Mayr E (2005) Low-intensity ultrasound enhances maturation of callus after segmental transport. Clin Orthop Relat Res 430:189–194

Claes L, Augat P, Schorlemmer S et al (2008) Temporary distraction and compression of a diaphyseal osteotomy accelerates bone healing. J Orthop Res 26:772–777

Clements JR, Carpenter BB, Pourciau JK (2008) Treating segmental bone defects: a new technique. J Foot Ankle Surg 47:350–356

Dai KR, Xu XL, Tang TT et al (2005) Repairing of goat tibial bone defects with BMP-2 gene-modified tissue-engineered bone. Calcif Tissue Int 77:55–61

de Kleer V (2006) Development of bone. In: Sumner-Smith G (ed) Bone in clinical orthopedics. W.B. Saunders Co, Philadelphia, pp 1–80

de Pablos J, Barrios C, Alfaro C et al (1994) Large experimental segmental bone defects treated by bone transportation with monolateral external distractors. Clin Orthop Relat Res 298:259–265

DeCoster TA, Gehlert RJ, Mikola EA et al (2004) Management of posttraumatic segmental bone defects. J Am Acad Orthop Surg 12:28–38

Dell PC, Burchardt H, Glowczewskie FP Jr (1985) A roentgenographic, biomechanical, and histological evaluation of vascularized and non-vascularized segmental fibular canine autografts. J Bone Joint Surg Am 67:105–112

den Boer FC, Patka P, Bakker FC et al (1999) New segmental long bone defect model in sheep: quantitative analysis of healing with dual energy x-ray absorptiometry. J Orthop Res 17:654–660

den Boer FC, Bramer JA, Blokhuis TJ et al (2002) Effect of recombinant human osteogenic protein-1 on the healing of a freshly closed diaphyseal fracture. Bone 31:158–164

den Boer FC, Wippermann BW, Blokhuis TJ et al (2003) Healing of segmental bone defects with granular porous hydroxyapatite augmented with recombinant human osteogenic protein-1 or autologous bone marrow. J Orthop Res 21:521–528

Di Bella C, Aldini NN, Lucarelli E et al (2010) Osteogenic protein-1 associated with mesenchymal stem cells promote bone allograft integration. Tissue Eng A 16:2967–2976

Edwards RB 3rd, Seeherman HJ, Bogdanske JJ et al (2004) Percutaneous injection of recombinant human bone morphogenetic protein-2 in a calcium phosphate paste accelerates healing of a canine tibial osteotomy. J Bone Joint Surg Am 86-A:1425–1438

Egermann M, Goldhahn J, Schneider E (2005) Animal models for fracture treatment in osteoporosis. Osteoporos Int 16(Suppl 2):S129–138

Einhorn TA (1999) Clinically applied models of bone regeneration in tissue engineering research. Clin Orthop Relat Res 367:S59–S67

Einhorn TA, Lane JM, Burstein AH et al (1984) The healing of segmental bone defects induced by demineralized bone matrix: a radiographic and biomechanical study. J Bone Joint Surg Am 66:274–279

Eitel F, Klapp F, Jacobson W et al (1981) Bone regeneration in animals and in man: a contribution to understanding the relative value of animal experiments to human pathophysiology. Arch Orthop Trauma Surg 99:59–64
Epari DR, Schell H, Bail HJ et al (2006) Instability prolongs the chondral phase during bone healing in sheep. Bone 38:864–870
Epari DR, Lienau J, Schell H et al (2008) Pressure, oxygen tension and temperature in the periosteal callus during bone healing-an in vivo study in sheep. Bone 43(4):734–739
Faria ML, Lu Y, Heaney K et al (2007) Recombinant human bone morphogenetic protein-2 in absorbable collagen sponge enhances bone healing of tibial osteotomies in dogs. Vet Surg 36:122–131
Field JR, McGee M, Wildenauer C et al (2009) The utilization of a synthetic bone void filler (JAX) in the repair of a femoral segmental defect. Vet Comp Orthop Traumatol 22:87–95
Field JR, McGee M, Stanley R et al (2011) The efficacy of allogeneic mesenchymal precursor cells for the repair of an ovine tibial segmental defect. Vet Comp Orthop Traumatol 24:113–121
Gao TJ, Lindholm TS, Kommonen B et al (1996) Enhanced healing of segmental tibial defects in sheep by a composite bone substitute composed of tricalcium phosphate cylinder, bone morphogenetic protein, and type IV collagen. J Biomed Mater Res 32:505–512
Gao TJ, Lindholm TS, Kommonen B et al (1997) The use of a coral composite implant containing bone morphogenetic protein to repair a segmental tibial defect in sheep. Int Orthop 21:194–200
Gazdag AR, Lane JM, Glaser D et al (1995) Alternatives to autogenous bone graft: efficacy and indications. J Am Acad Orthop Surg 3:1–8
Gerhart TN, Kirker-Head CA, Kriz MJ et al (1993) Healing segmental femoral defects in sheep using recombinant human bone morphogenetic protein. Clin Orthop Relat Res 293:317–326
Gilbert JA, Dahners LE, Atkinson MA (1989) The effect of external fixation stiffness on early healing of transverse osteotomies. J Orthop Res 7:389–397
Gillett N, Brown SA, Dumbleton JH et al (1985) The use of short carbon fibre reinforced thermoplastic plates for fracture fixation. Biomaterials 6:113–121
Goldstrohm GL, Mears DC, Swartz WM (1984) The results of 39 fractures complicated by major segmental bone loss and/or leg length discrepancy. J Trauma 24:50–58
Gong JK, Arnold JS, Cohn SH (1964) The density of organic and volatile and non-volatile inorganic components of bone. Anat Rec 149:319–324
Goodship AE, Watkins PE, Rigby HS et al (1993) The role of fixator frame stiffness in the control of fracture healing: an experimental study. J Biomech 26:1027–1035
Gotterbarm T, Breusch SJ, Schneider U et al (2008) The minipig model for experimental chondral and osteochondral defect repair in tissue engineering: retrospective analysis of 180 defects. Lab Anim 42:71–82
Gray AC, White TO, Clutton E et al (2009) The stress response to bilateral femoral fractures: a comparison of primary intramedullary nailing and external fixation. J Orthop Trauma 23:90–97 (discussion 98–99)
Gugala Z, Gogolewski S (1999) Regeneration of segmental diaphyseal defects in sheep tibiae using resorbable polymeric membranes: a preliminary study. J Orthop Trauma 13:187–195
Gugala Z, Gogolewski S (2002) Healing of critical-size segmental bone defects in the sheep tibiae using bioresorbable polylactide membranes. Injury 33(suppl 2):B71–B76
Gugala Z, Lindsey RW, Gogolewski S (2007) New approaches in the treatment of critical-size segmental defects in long bones. Macromol Symp 253:147–161
Hantes ME, Mavrodontidis AN, Zalavras CG et al (2004) Low-intensity transosseous ultrasound accelerates osteotomy healing in a sheep fracture model. J Bone Joint Surg Am 86-A:2275–2282
Hara Y, Nakamura T, Fukuda H et al (2003) Changes of biomechanical characteristics of the bone in experimental tibial osteotomy model in the dog. J Vet Med Sci 65:103–107
Heitemeyer U, Claes L, Hierholzer G (1990) The significance of postoperative stability for osseous repair of a multiple fragment fracture. Animal experiment studies. Unfallchirurg 93:49–55
Hente R, Cordey J, Rahn BA et al (1999) Fracture healing of the sheep tibia treated using a unilateral external fixator: comparison of static and dynamic fixation. Injury 30(suppl 1):A44–A51

Hill PF, Watkins PE (2001) The prevention of experimental osteomyelitis in a model of gunshot fracture in the pig. Eur J Orthop Surg Traumatol 11:237–241

Huang J, Zhang L, Chu B et al (2011) Repair of bone defect in caprine tibia using a laminated scaffold with bone marrow stromal cells loaded poly (L-lactic acid)/beta-tricalcium phosphate. Artif Organs 35:49–57

Hupel TM, Weinberg JA, Aksenov SA et al (2001) Effect of unreamed, limited reamed, and standard reamed intramedullary nailing on cortical bone porosity and new bone formation. J Orthop Trauma 15:18–27

Ilizarov GA (1989) The tension-stress effect on the genesis and growth of tissues: part II: the influence of the rate and frequency of distraction. Clin Orthop Relat Res 239:263–285

Ilizarov GA, Gracheva VI (1971) Bloodless treatment of congenital pseudarthrosis of the crus with simultaneous elimination of shortening using dosed distraction. Ortop Travmatol Protez 32:42–46

Itoh S, Kikuchi M, Takakuda K et al (2002) Implantation study of a novel hydroxyapatite/collagen (HAp/col) composite into weight-bearing sites of dogs. J Biomed Mater Res 63:507–515

Jain R, Podworny N, Hupel TM et al (1999) Influence of plate design on cortical bone perfusion and fracture healing in canine segmental tibial fractures. J Orthop Trauma 13:178–186

Jiang CC, Chiang H, Liao CJ et al (2007) Repair of porcine articular cartilage defect with a biphasic osteochondral composite. J Orthop Res 25:1277–1290

Jockisch KA, Brown SA, Bauer TW et al (1992) Biological response to chopped-carbon-fiber-reinforced peek. J Biomed Mater Res 26:133–146

Kettunen J, Makela A, Miettinen H et al (1999) Fixation of femoral shaft osteotomy with an intramedullary composite rod: an experimental study on dogs with a two-year follow-up. J Biomater Sci Polym Ed 10:33–45

Kilian O, Wenisch S, Alt V et al (2007) Effects of platelet factors on biodegradation and osteogenesis in metaphyseal defects filled with nanoparticular hydroxyapatite – an experimental study in minipigs. Growth Factors 25:191–201

Kirker-Head CA, Gerhart TN, Armstrong R et al (1998) Healing bone using recombinant human bone morphogenetic protein 2 and copolymer. Clin Orthop Relat Res 349:205–217

Klein C, Sprecher C, Rahn BA et al (2010) Unreamed or RIA reamed nailing: an experimental sheep study using comparative histological assessment of affected bone tissue in an acute fracture model. Injury 41(suppl 2):S32–S37

Kleinman PL, Zurakowski D, Strauss KJ et al (2008) Detection of simulated inflicted metaphyseal fractures in a fetal pig model: image optimization and dose reduction with computed radiography. Radiology 247:381–390

Knothe Tate ML, Ritzman TF, Schneider E et al (2007) Testing of a new one-stage bone-transport surgical procedure exploiting the periosteum for the repair of long-bone defects. J Bone Joint Surg Am 89:307–316

Knothe Tate ML, Chang H, Moore SR et al (2011) Surgical membranes as directional delivery devices to generate tissue: testing in an ovine critical sized defect model. PloS One 6:e28702

Kokubo S, Mochizuki M, Fukushima S et al (2004) Long-term stability of bone tissues induced by an osteoinductive biomaterial, recombinant human bone morphogenetic protein-2 and a biodegradable carrier. Biomaterials 25:1795–1803

Komaki H, Tanaka T, Chazono M et al (2006) Repair of segmental bone defects in rabbit tibiae using a complex of beta-tricalcium phosphate, type I collagen, and fibroblast growth factor-2. Biomaterials 27:5118–5126

Kuttenberger JJ, Stubinger S, Waibel A et al (2008) Computer-guided CO2-laser osteotomy of the sheep tibia: technical prerequisites and first results. Photomed Laser Surg 26:129–136

Laurencin C, Khan Y, El-Amin SF (2006) Bone graft substitutes. Expert Rev Med Devices 3:49–57

Lian ZD, Chuanchang L, Wei et al (2009) Enhanced healing of goat femur-defect using BMP7 gene-modified BMSCs and load-bearing tissue-engineered bone. J Orthop Res 28:412–418

Liebschner MA (2004) Biomechanical considerations of animal models used in tissue engineering of bone. Biomaterials 25:1697–1714

Lindsey RW, Gugala Z, Milne E et al (2006) The efficacy of cylindrical titanium mesh cage for the reconstruction of a critical-size canine segmental femoral diaphyseal defect. J Orthop Res 24:1438–1453

Liu G, Zhao L, Zhang W et al (2008) Repair of goat tibial defects with bone marrow stromal cells and beta-tricalcium phosphate. J Mater Sci Mater Med 19:2367–2376

Liu X, Li X, Fan Y et al (2010) Repairing goat tibia segmental bone defect using scaffold cultured with mesenchymal stem cells. Journal of biomedical materials research. Part B. J Biomed Mater Res B Appl Biomater 94:44–52

Lu Y, Nemke B, Lorang DM et al (2009) Comparison of a new braid fixation system to an interlocking intramedullary nail for tibial osteotomy repair in an ovine model. Vet Surg 38:467–476

Macdonald W, Skirving AP, Scull ER (1988) A device for producing experimental fractures. Acta Orthop Scand 59:542–544

Maissen O, Eckhardt C, Gogolewski S et al (2006) Mechanical and radiological assessment of the influence of rhTGFbeta-3 on bone regeneration in a segmental defect in the ovine tibia: pilot study. J Orthop Res 24:1670–1678

Manjubala I, Liu Y, Epari DR et al (2009) Spatial and temporal variations of mechanical properties and mineral content of the external callus during bone healing. Bone 45:185–192

Markel MD, Wikenheiser MA, Chao EY (1990) A study of fracture callus material properties: relationship to the torsional strength of bone. J Orthop Res 8:843–850

Martini L, Fini M, Giavaresi G et al (2001) Sheep model in orthopedic research: a literature review. Comp Med 51:292–299

Mastrogiacomo M, Corsi A, Francioso E et al (2006) Reconstruction of extensive long bone defects in sheep using resorbable bioceramics based on silicon stabilized tricalcium phosphate. Tissue Eng 12:1261–1273

Meinel L, Zoidis E, Zapf J et al (2003) Localized insulin-like growth factor I delivery to enhance new bone formation. Bone 33:660–672

Miettinen H, Makela EA, Vainio J et al (1992) The effect of an intramedullary self-reinforced poly-L-lactide (SR-PLLA) implant on growing bone with special reference to fixation properties: an experimental study on growing rabbits. J Biomater Sci Polym Ed 3:443–450

Mosekilde L, Weisbrode SE, Safron JA et al (1993) Calcium-restricted ovariectomized Sinclair S-1 minipigs: an animal model of osteopenia and trabecular plate perforation. Bone 14: 379–382

Mousavi M, David R, Schwendenwein I et al (2002) Influence of controlled reaming on fat intravasation after femoral osteotomy in sheep. Clin Orthop Relat Res 394:263–270

Muscolo DL, Ayerza MA, Aponte-Tinao LA (2006) Massive allograft use in orthopedic oncology. Orthop Clin North Am 37:65–74

Nafei A, Danielsen CC, Linde F et al (2000) Properties of growing trabecular ovine bone. Part I: mechanical and physical properties. J Bone Joint Surg Br 82:910–920

Nair MB, Varma HK, Menon KV et al (2009) Reconstruction of goat femur segmental defects using triphasic ceramic-coated hydroxyapatite in combination with autologous cells and platelet-rich plasma. Acta Biomater 5:1742–1755

Nakamura T, Hara Y, Tagawa M et al (1998) Recombinant human basic fibroblast growth factor accelerates fracture healing by enhancing callus remodeling in experimental dog tibial fracture. J Bone Miner Res 13:942–949

Newman E, Turner AS, Wark JD (1995) The potential of sheep for the study of osteopenia: current status and comparison with other animal models. Bone 16:277S–284S

Neyt JG, Buckwalter JA, Carroll NC (1998) Use of animal models in musculoskeletal research. Iowa Orthop J 18:118–123

Niemeyer P, Schonberger TS, Hahn J et al (2009) Xenogenic transplantation of human mesenchymal stem cells in a critical size defect of the sheep tibia for bone regeneration. Tissue Eng A 16:33–43

Niemeyer P, Fechner K, Milz S et al (2010) Comparison of mesenchymal stem cells from bone marrow and adipose tissue for bone regeneration in a critical size defect of the sheep tibia and the influence of platelet-rich plasma. Biomaterials 31:3572–3579

O'Loughlin PF, Morr S, Bogunovic L et al (2008) Selection and development of preclinical models in fracture-healing research. J Bone Joint Surg Am 90(suppl 1):79–84
Oest ME, Dupont KM, Kong HJ et al (2007) Quantitative assessment of scaffold and growth factor-mediated repair of critically sized bone defects. J Orthop Res 25:941–950
Pearce AI, Richards RG, Milz S et al (2007) Animal models for implant biomaterial research in bone: a review. Eur Cell Mater 13:1–10
Pek YS, Gao S, Arshad MS et al (2008) Porous collagen-apatite nanocomposite foams as bone regeneration scaffolds. Biomaterials 29:4300–4305
Perka C, Schultz O, Spitzer RS et al (2000) Segmental bone repair by tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. Biomaterials 21:1145–1153
Perry CR (1999) Bone repair techniques, bone graft, and bone graft substitutes. Clin Orthop Relat Res 360:71–86
Petite H, Viateau V, Bensaid W et al (2000) Tissue-engineered bone regeneration. Nat Biotechnol 18:959–963
Puelacher WC, Vacanti JP, Ferraro NF et al (1996) Femoral shaft reconstruction using tissue-engineered growth of bone. Int J Oral Maxillofac Surg 25:223–228
Rahal SC, Volpi RS, Vulcano LC (2005) Treatment of segmental tibial defects using acute bone shortening followed by gradual lengthening with circular external fixator. J Vet Med A Physiol Pathol Clin Med 52:180–185
Raschke M, Kolbeck S, Bail H et al (2001) Homologous growth hormone accelerates healing of segmental bone defects. Bone 29:368–373
Ravaglioli A, Krajewski A, Celotti GC et al (1996) Mineral evolution of bone. Biomaterials 17:617–622
Reichert JC, Epari DR, Wullschleger ME et al (2010) Establishment of a preclinical ovine model for tibial segmental bone defect repair by applying bone tissue engineering strategies. Tissue Eng Part B Rev 16:93–104
Reichert JC, Wullschleger ME, Cipitria A et al (2011) Custom-made composite scaffolds for segmental defect repair in long bones. Int Orthop 35:1229–1236
Reichert JC, Epari DR, Wullschleger ME et al (2012) Bone tissue engineering: reconstruction of critical sized segmental bone defects in the ovine tibia. Orthopade 41:280–287
Rimondini L, Nicoli-Aldini N, Fini M et al (2005) In vivo experimental study on bone regeneration in critical bone defects using an injectable biodegradable PLA/PGA copolymer. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 99:148–154
Rozen N, Bick T, Bajayo A et al (2009) Transplanted blood-derived endothelial progenitor cells (EPC) enhance bridging of sheep tibia critical size defects. Bone 45:918–924
Sarkar MR, Augat P, Shefelbine SJ et al (2006) Bone formation in a long bone defect model using a platelet-rich plasma-loaded collagen scaffold. Biomaterials 27:1817–1823
Schell H, Epari DR, Kassi JP et al (2005) The course of bone healing is influenced by the initial shear fixation stability. J Orthop Res 23:1022–1028
Schemitsch EH, Kowalski MJ, Swiontkowski MF et al (1994) Cortical bone blood flow in reamed and unreamed locked intramedullary nailing: a fractured tibia model in sheep. J Orthop Trauma 8:373–382
Schemitsch EH, Kowalski MJ, Swiontkowski MF (1996) Soft-tissue blood flow following reamed versus unreamed locked intramedullary nailing: a fractured sheep tibia model. Ann Plast Surg 36:70–75
Schimandle JH, Boden SD (1994) Spine update: the use of animal models to study spinal fusion. Spine 19:1998–2006
Schneiders W, Reinstorf A, Biewener A et al (2008) In vivo effects of modification of hydroxyapatite/collagen composites with and without chondroitin sulphate on bone remodeling in the sheep tibia. J Orthop Res 27:15–21
Schnettler R, Alt V, Dingeldein E et al (2003) Bone ingrowth in bFGF-coated hydroxyapatite ceramic implants. Biomaterials 24:4603–4608

Sciadini MF, Dawson JM, Johnson KD (1997) Evaluation of bovine-derived bone protein with a natural coral carrier as a bone-graft substitute in a canine segmental defect model. J Orthop Res 15:844–857

Skirving AP, Day R, Macdonald W et al (1987) Carbon fiber reinforced plastic (CFRP) plates versus stainless steel dynamic compression plates in the treatment of fractures of the tibiae in dogs. Clin Orthop Relat Res 224:117–124

Starr AJ, Welch RD, Eastridge BJ et al (2002) The effect of hemorrhagic shock in a caprine tibial fracture model. J Orthop Trauma 16:250–256

Stevenson S (1998) Enhancement of fracture healing with autogenous and allogeneic bone grafts. Clin Orthop Relat Res 355:S239–S246

Sun C, Huang G, Christensen FB et al (1999) Mechanical and histological analysis of bone-pedicle screw interface in vivo: titanium versus stainless steel. Chin Med J (Engl) 112:456–460

Takigami H, Kumagai K, Latson L et al (2007) Bone formation following OP-1 implantation is improved by addition of autogenous bone marrow cells in a canine femur defect model. J Orthop Res 25:1333–1342

Taylor GI, Miller GD, Ham FJ (1975) The free vascularized bone graft: a clinical extension of microvascular techniques. Plast Reconstr Surg 55:533–544

Taylor WR, Ehrig RM, Duda GN et al (2005) On the influence of soft tissue coverage in the determination of bone kinematics using skin markers. J Orthop Res 23:726–734

Taylor WR, Ehrig RM, Heller MO et al (2006) Tibio-femoral joint contact forces in sheep. J Biomech 39:791–798

Teixeira CR, Rahal SC, Volpi RS et al (2007) Tibial segmental bone defect treated with bone plate and cage filled with either xenogeneic composite or autologous cortical bone graft: an experimental study in sheep. Vet Comp Orthop Traumatol 20:269–276

Tepic S, Remiger AR, Morikawa K et al (1997) Strength recovery in fractured sheep tibia treated with a plate or an internal fixator: an experimental study with a two-year follow-up. J Orthop Trauma 11:14–23

Thorwarth M, Schultze-Mosgau S, Kessler P et al (2005) Bone regeneration in osseous defects using a resorbable nanoparticular hydroxyapatite. J Oral Maxillofac Surg 63:1626–1633

Tiedeman JJ, Lippiello L, Connolly JF et al (1990) Quantitative roentgenographic densitometry for assessing fracture healing. Clin Orthop Relat Res 253:279–286

Tseng SS, Lee MA, Reddi AH (2008) Nonunions and the potential of stem cells in fracture-healing. J Bone Joint Surg Am 90(Suppl 1):92–98

Tyllianakis M, Deligianni D, Panagopoulos A et al (2007) Biomechanical comparison of callus over a locked intramedullary nail in various segmental bone defects in a sheep model. Med Sci Monit 13:BR125–BR130

van der Elst M, Klein CP, de Blieck-Hogervorst JM et al (1999) Bone tissue response to biodegradable polymers used for intra medullary fracture fixation: a long-term in vivo study in sheep femora. Biomaterials 20:121–128

Viateau V, Guillemin G, Yang YC et al (2004) A technique for creating critical-size defects in the metatarsus of sheep for use in investigation of healing of long-bone defects. Am J Vet Res 65:1653–1657

Viateau V, Guillemin G, Calando Y et al (2006) Induction of a barrier membrane to facilitate reconstruction of massive segmental diaphyseal bone defects: an ovine model. Vet Surg 35:445–452

Viateau V, Guillemin G, Bousson V et al (2007) Long-bone critical-size defects treated with tissue-engineered grafts: a study on sheep. J Orthop Res 25:741–749

Wallace AL, Makki R, Weiss JB et al (1995) Measurement of serum angiogenic factor in devascularized experimental tibial fractures. J Orthop Trauma 9:324–332

Wang X, Mabrey JD, Agrawal CM (1998) An interspecies comparison of bone fracture properties. Biomed Mater Eng 8:1–9

Wang CJ, Huang HY, Chen HH et al (2001) Effect of shock wave therapy on acute fractures of the tibia: a study in a dog model. Clin Orthop Relat Res 387:112–118

Wefer J, Wefer A, Schratt HE et al (2000) Healing of autologous cancellous bone transplants and hydroxylapatite ceramics in tibial segment defects: value of ultrasonic follow up. Unfallchirurg 103:452–461

Welch RD, Jones AL, Bucholz RW et al (1998) Effect of recombinant human bone morphogenetic protein-2 on fracture healing in a goat tibial fracture model. J Bone Miner Res 13:1483–1490

Wildemann B, Kadow-Romacker A, Pruss A et al (2007) Quantification of growth factors in allogenic bone grafts extracted with three different methods. Cell Tissue Bank 8:107–114

Wilson DJ, Morgan RL, Hesselden KL et al (2009) A single-channel telemetric intramedullary nail for in vivo measurement of fracture healing. J Orthop Trauma 23:702–709

Windhagen H, Kolbeck S, Bail H et al (2000) Quantitative assessment of in vivo bone regeneration consolidation in distraction osteogenesis. J Orthop Res 18:912–919

Younger EM, Chapman MW (1989) Morbidity at bone graft donor sites. J Orthop Trauma 3:192–195

Zhu L, Liu W, Cui L et al (2006) Tissue-engineered bone repair of goat-femur defects with osteogenically induced bone marrow stromal cells. Tissue Eng 12:423–433

Zhu L, Chuanchang D, Wei L et al (2010) Enhanced healing of goat femur-defect using BMP7 gene-modified BMSCs and load-bearing tissue-engineered bone. J Orthop Res 28:412–418

Chapter 41
Constraints to Articular Cartilage Regeneration

Georg N. Duda, Joshua O. Eniwumide, and Michael Sittinger

Abstract The glassy translucent material found at the ends of bones, within synovial joints, is termed articular cartilage. While healthy, it provides a low-friction bearing surface, preventing bone-to-bone contact, and to an extent, absorb shock during vigorous activities. However, when damaged could lead to pain, deformity and reduced mobility; the social impact of which, entails high costs in terms of therapeutic treatments and loss of income. The present chapter reviews the common knowledge of the constraints to articular cartilage regeneration; namely cartilage structure, composition and major diseases. The first of the three sections detail the major constituents of the tissue and their structural organisation; the tissues mechanical properties, and ends with a brief description of how these features change in an unhealthy cartilage; be it mechanical or disease. In the second section, both clinical and academic approaches are pooled together, to review the current strategies in restoring health to joints with diseased or damaged cartilage. The final section highlights the fact that progression of cartilage disease affects not only the cartilage, but its underlying bone. The implications of the subchondral bone in the propagation of cartilage degeneration are discussed, and finally, their considerations in cartilage defect healing.

G.N. Duda, Ph.D. (✉) • J.O. Eniwumide, Ph.D.
Julius Wolff Institute and Centre for Musculoskeletal Surgery, Berlin-Brandenburg Centre for Regenerative Therapy, Charité, Augustenburgerplatz 1, 13353 Berlin, Germany
e-mail: georg.duda@charite.de

M. Sittinger, Ph.D.
Department of Rheumatology and Clinical Immunology, Tissue Engineering Laboratory and Berlin-Brandenburg Center for Regenerative Therapies, Charité-Universitätsmedizin Berlin, Tucholskystrasse 2, 10117 Berlin, Germany

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_41,

41.1 In Health and Sickness

41.1.1 Structure and Function

Articular cartilage is found at the end of bones as a thin, white tissue. This tissue consists mainly of extracellular matrix (ECM), and has a relatively low density of cells. The adult tissue is known to be devoid of blood vessels, lymph vessels, and nerves (Poole 1997; Stockwell and Meachim 1979). The thickness of adult human cartilage generally varies between 2 and 7 mm (Meachim and Stockwell 1979). This variation is evident both between joints and also within different regions of the same joint.

The efficient functioning of the synovial joints is made possible by the presence of articular cartilage. Lining the surfaces of diarthrodial joints, articular cartilage provides a low-friction bearing surface and prevents bone-to-bone contact (Guilak and Mow 2000; Mow et al. 1980). Major load bearing joints, such as the hip, knee and ankle are subjected to peak stresses up to three times body weight during normal walking or higher during stumbling (Guilak and Mow 2000; Mow et al. 1992; Weightman and Kempson 1979). Under these high stresses, articular cartilage deforms, effectively reducing both the contact stresses and the pressures transmitted to the underlying bone (Ateshian and Wang 1997; Kim et al. 1994; McCutchen 1962; Weightman and Kempson 1979). Cartilage also exhibits impact resistance, which permits a degree of shock absorbance during vigorous activities such as running and jumping.

41.1.2 Composition

Articular cartilage consists of Water, Collagen, and non-collageneous proteins, and cells. These are approximated to be 70–80, 10–15, and 5–10% of the tissues wet weight, respectively, while the cells are approximately 5% of the tissues volume. Notably these features vary amongst species, from joint to joint and within different locations of the same joint (Buckwalter and Mankin 1997; Stockwell and Meachim 1979).

41.1.2.1 Collagen

Collagen is a large protein family with at least 27 members (Boot-Handford et al. 2003; Eyre 2004). They can be distinguished by their distinct amino acid composition and hence polypeptide chains (Meisenberg and Simmons 1998). The collagen types present in articular cartilage are types II, VI, IX, X and XI (Eyre et al. 1992). Type II collagen makes up 95% of the solid composition of the mature human articular cartilage (Eyre et al. 1992). It is derived from procollagen molecules containing

amino (NH_2)-and carboxyl (COOH)-terminal extension peptides that are cleaved, extracellular, prior to fibrilcollagens (Ryan and Sandell 1990).

Due to their abundance in articular cartilage, type II and type X collagen are often used as indicators for extracellular matrix (ECM) formation by cultured chondrocytes and to ascertain a chondrogenic phenotype of differentiating stem cells.

Type XI collagen contributes less than 5% to the total collagen content of the articular cartilage. In cartilaginous tissues, collagen XI forms heterotypic narrow fibrils with collagens type II and type IX (Grant et al. 1988). Type VI collagen is a short-helical heterotrimer. Its monomers are arranged intracellularly into anti-parallel staggered dimers and then into tetramers by lateral aggregation of two dimmers, then secreted into the ECM. In the pericellular environment, collagen VI has been implicated in both the maintenance of chondron integrity and cell-matrix signalling. Type IX collagen accounts for 10% of human foetal cartilage. This proportion decreases with age, reaching 1–2% in mature human articular cartilage. This molecule can be considered a proteoglycan, due to its possession of a chondroitin sulphate chain (Huber et al. 1988). Type X collagen is a short-chain collagen, which forms a mat-like network in the hypertrophic cartilage matrix and around differentiating chondrocytes (Kwan et al. 1986). It is found in either the cartilaginous tissues undergoing endochondral ossification, such as the hypertrophic zones of the growth plate, or the calcified zone in mature articular cartilage (Ayad et al. 1987).

41.1.2.2 Proteoglycans

Proteoglycans (PGs) are the most abundant non-collageneous macromolecular components in mammalian cartilage, making up approximately 10–15% of the mature mammalian articular cartilage. These are defined as having a protein core to which one or more glycosaminoglycan (GAG) chains are covalently attached. GAGs present in articular cartilage include chondroitin sulphate, keratan sulphate and hyaluronan. The GAG chains are often crucial to the functional properties of the PG (Hardingham and Fosang 1992). However, for PGs such as decorin and biglycan, which bind to growth factors and modulate their activities, evidence exist, suggesting that it is their protein core and not their GAG chains that mediates the binding function (Cheifetz et al. 1988; Ruoslahti and Yamaguchi 1991). The most abundant PG, accounting for up to 90% of mature articular cartilage PG is aggrecan. As GAG builds up in the cells ECM, aggrecan molecules bind non-covalently along a hyaluronan chain to form an aggrecan-hyaluronan complex. The aggregate, having molecular weight approximately 50,000 kDa is associated with load distribution in articular cartilage (Hardingham and Fosang 1992). Smaller PG molecules present in cartilage include biglycan, decorin and fibromodulin (Knudson and Knudson 2001). These leucine-rich PGs bind to collagen type II and play important roles in ECM organisation (Pulkkinen et al. 1990; Vogel et al. 1984).

PGs have important roles in collagen fibrillogenesis, organisation of collagen networks and in providing rigidity to the ECM. Chondrocytes express cell surface PGs (Knudson and Knudson 2001), which interact with growth factors such as,

basic Fibroblastic Growth Factor (bFGF) in order to regulate cell activities. Similar to types II and X collagen, PG provide a reliable indicator of biosynthetic activities of chondrocytes in culture (Knight et al. 1998; Lee et al. 2003). Other common examples of such proteins are Anchorin CII. These bind to the surface of the chondrocytes and anchor it to the collagen fibrils in the ECM and cartilage oligomeric matrix proteins, which also bind to chondrocytes (Mollenhauer et al. 1984). These proteins maintain the chondrocytes phenotype, and are used as markers of cartilage turnover and can control the progression of cartilage degradation in osteoarthritic cartilage (Salter 1993). Other proteins, such as fibronectin and tenascin have roles in matrix organisation, cell-matrix interaction and the tissue response to inflammatory conditions such as osteoarthritis (Buckwalter and Mankin 1997; Nishida et al. 1995).

41.1.2.3 Extracellular Matrix Fluid

Water is the largest component of articular cartilage. It makes up to 80% of cartilage wet weight in the surface zone, and decreases to approximately 65% within the deep zone. The high affinity of articular cartilage for water is due to the charge density of the hydrophilic PG molecules. The PGs encapsulate the water within their matrices, forming a gel-like substance, with pore size of approximately 6.9 nm (Meachim and Stockwell 1979). These pores contribute to the diffusivity of the small molecules and water through articular cartilage (Lusse et al. 2000; Maroudas 1979). Matrix water contains small gasses, proteins and dissolved electrolytes (Na^+, Cl^-, Ca^{2+}, SO_3^-, COO^- etc.). The cations balance the negatively charged PGs, thereby influencing the mechanical properties of the tissue (Guilak et al. 1999). A large proportion of the ECM fluid can move freely in and out of the tissue under applied load (Buckwalter and Mankin 1997). Therefore during normal joint loading, the cartilage is compressed and the water is squeezed out of the loaded region. As the region is unloaded, the water is re-imbibed and the original volume is restored with time. This movement of cartilage fluid is crucial in joint lubrication, transport of macromolecules within articular cartilage and nutrition of the cells therein.

41.1.2.4 Chondrocytes

The cartilage ECM and its associated proteins are synthesised, assembled and organised into a highly ordered framework by its cellular component, the articular chondrocytes (Buckwalter and Mankin 1997; Muir 1995). The chondrocytes attempt to maintain the ECM and their associated protein by their continual replacement in health, disease and following trauma. However, this depends on the cells ability to detect changes in the matrix composition, which may be due to macromolecular degradation, or the mechanical demands placed upon the tissue. Although relatively sparse in density, the chondrocytes are the only living units available to adapt cartilage

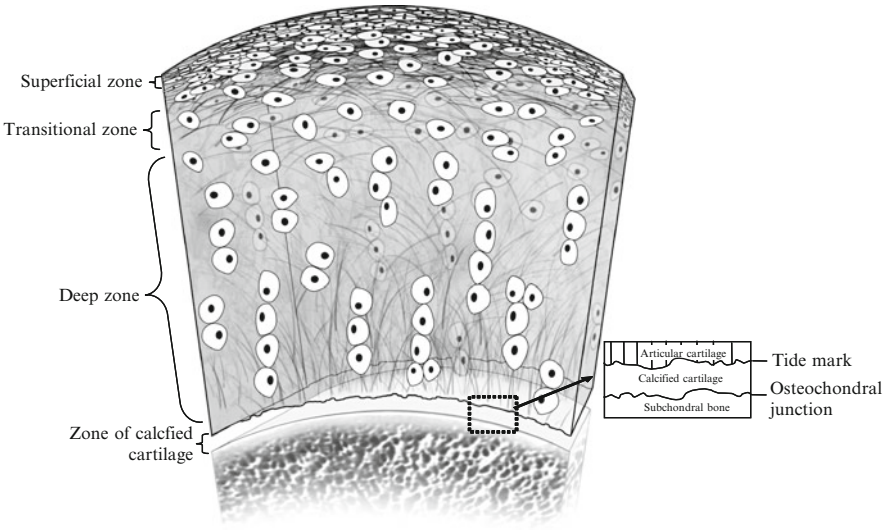


Fig. 41.1 Structure and composition of cartilage at different depths from the articular surface

to changes in its surroundings. Individually, chondrocytes have been found to be metabolically active, with a glycolytic rate per cell similar to that of cells found in vascularised tissues. However, the adult tissue as a whole, has a comparatively low metabolic activity due to its low cell density of approximately 1 cell per 100 μm^3 (Buckwalter et al. 2005). Both cell proliferation and ECM synthesis decline following skeletal maturity in normal tissue.

41.1.3 Organisation

The structure and composition of articular cartilage changes with depth from the joint surface (Buckwalter et al. 1987; Clarke 1971; Lane and Weiss 1975; Lipshitz et al. 1976; Muir et al. 1970; Ratcliffe and Mow 1976). Although these changes are continuous, articular cartilage has been divided into four distinct zones/layers (Fig. 41.1). These are termed the superficial zone (I), transitional zone (II), the deep or radial zone (III), and the zone of calcified cartilage (IV). PGs occupy the interfibrillar space and their concentrations increase from the surface to a maximum in the transitional zone, and then diminishes toward the deep zone (Comper 1996; Muir 1980; Poole et al. 1982). The volumetric concentration of collagen fibres in human articular cartilage increases from the superficial (16–31%) to the deep zone (14–42%), while that of the cell decrease by a factor of about three from the surface to the deep zone. The superficial zone is closest to the articular surface. It is approximately 250 μm thick for human articular cartilage, and is the thinnest of the four

zones. Chondrocytes in this zone are flattened and oriented parallel to the articular surface (Meachim and Stockwell 1979). Collagen fibres in this region are very fine and, are arranged tangentially to the surface of the cartilage, thus deriving its alternative name; the tangential zone (Buckwalter et al. 1987). The most superficial part of this region is termed the lamina splendens and is devoid of cells, but consists of fine fibres and polysaccharides.

In the transitional zone, chondrocytes are spherical in form and fairly uniform in distribution. The collagen fibrils in this zone are generally larger and more randomly organised. There is a higher concentration of PGs and lower water content when compared with the superficial zone (Buckwalter and Mankin 1997). The deep zone has a thickness greater than 500 μm, making it the thickest of the four zones. Chondrocytes of the deep zone are spherical, and are arranged in columns of four to nine cells, oriented perpendicularly to the joint surface (Meachim and Stockwell 1979). Collagen fibres within the deep zone are arranged perpendicularly to the articular surface. The zone of calcified cartilage separates the radial zone and subchondral bone. The deep and the calcified zones are separated by the *tide mark*. It is widely believed that the calcified zone of articular cartilage and the *tide mark* present barriers for solute diffusion via the subchondral bone and therefore nutrition is solely from the synovial fluid, via the articular surface (Honner and Thompson 1971; McKibbin and Holdsworth 1966). Evidence does exist, however to suggest the contrary, in that molecules can travel across to the articular cartilage. Notably, most of these studies have used immature synovial joint, and it is generally accepted that the route for nutrient delivery to the articular cartilage is affected by skeletal maturity (Honner and Thompson 1971). Compared to the deep zones, cells in the calcified zone have a smaller volume, and are associated with fewer golgi membranes and endoplasmic reticula in the cytoplasm, thus suggesting a reduced metabolic activity (Buckwalter and Mankin 1997).

41.1.4 Cartilage Biomechanics

Mechanically, a healthy articular cartilage acts to limit the contact stresses acting on the underlying bone and provide an extremely efficient low wear bearing surface for smooth movement (Kempson et al. 1970; Mow et al. 1992). For these reasons, cartilage is more deformable than bone, and thus when loaded, can provide a considerable area of contact to support joint loads (Weightman and Kempson 1979).

Interactions between the two main solid components of the ECM have crucial roles in the tissues ability to sustain an applied load (Maroudas 1976; Wong et al. 2000). For example, PG exhibits a swelling capacity, resulting from the negatively charged GAG molecules repelling each other while attracting water and mobile cations. Therefore PG molecules impede the loss of water in the matrix by reducing the tissue's permeability (Quinn et al. 2001). When not loaded, the associated osmotic pressure is balanced by the hydrostatic pressure resulting from the tensile stresses within the network of collagen fibres.

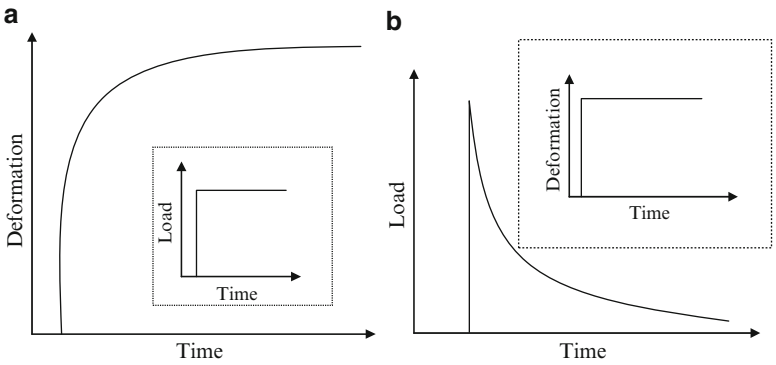


Fig. 41.2 (**a**) Creep and (**b**) load relaxation measurements of cartilage

41.1.4.1 Cartilage Loading

When cartilage is loaded, there is a short-lived response that alters the balance between the osmotic and hydrostatic pressures. The internal pressures increase within the joints, producing pressure gradients. Consequently, fluid flows away from the tissue, resulting in an increased in PG concentration (Weightman and Kempson 1979). To minimise this displacement, tensile stresses builds up in the collagen network. As the load is removed from the joint, fluid flows back into the cartilage and it regains its original shape. This response varies with the magnitude and type of load applied (Herzog et al. 1998).

During prolonged loading of cartilage, fluid flows out from the tissue and over time, there is a loss of volume, resulting in a time-dependant creep response. During static loading such as squatting and maintaining a 90° bend, the load transmission is confined to a relatively smaller area of the joint, the contact stress and deformation in this region is considerably greater than anywhere else the joint. Despite the loading rate being lower than that in knee bending exercises, the cartilage is loaded continuously at a local site over a long period, and this can lead to creep-behaviour (Eckstein et al. 2000).

41.1.4.2 Creep

The mechanical properties of cartilage can be measured either in creep or stress relaxation. Creep is the slow time-dependent deformation process which occurs after the immediately, elastic deformation of cartilage during load application (Fig. 41.2a). This is then followed by a slow, time dependant increased deformation. The initial deformation is brought about by the tissues' matrix, thus there is no net change to its volume. Moreover, the tissues resistance to deformation is due to the network of collagen fibres. However, the time-dependent deformation, which

occurs as the load is maintained, results directly from the imbibition of water from the tissues matrix. As the fluid leaves cartilage, the applied load is transferred from the matrix fluid to the solid components. An equilibrium is established when the load is totally transferred unto the matrix fibres. For creep measurement of cartilage, the tissue is compressed with a constant load and the resulting deformation is recorded.

41.1.4.3 Load Relaxation

In load relaxation, cartilage is compressed to a constant deformation and the load is measured (Fig. 41.2b). Initially, the load required to maintain a constant deformation is high. This is necessary in order to pressurise the matrix fluid. As the fluid begins to leave the tissue, the load required to maintain the deformation decreases and tend towards a plateau. At this stage, the matrix is compacted and the interfibrilar pore space is reduced. Equilibrium is then established when fluid is no longer exiting the cartilage, and the remaining fluid is redistributed within the tissue. Both the time-dependent creep and load relaxation of cartilage is largely due to the fluid flow, whereas the equilibrium is controlled by the solid matrix. In fact, the equilibrium stiffness of articular cartilage has been tied to the tissues PG content (Jurvelin et al. 1988; Mow et al. 1980).

41.1.4.4 *In Vitro* Mechanical Testing

Three common methods used for determining the mechanical properties of cartilage are confined compression, unconfined compression and indentation testing. In confined compression (Fig. 41.3a), cartilage is placed a non-porous chamber and compressed with a porous platen, so fluid is forced out only via the porous platen. During unconfined compression (Fig. 41.3b) however, cartilage is compressed between two non-porous platens and fluid exits laterally. For indentation tests (Fig. 41.3c), cartilage is compressed with an indenter that is either porous or non porous. In cases where a porous indenter is used, fluid expelled from the cartilage may flow laterally or axially. However, non-porous indenters impede axial fluid flow.

Although common mechanical parameters may be obtained using any of the three strategies, it has been recognised that the values of mechanical properties are dependent on the measurement technique employed. For example, Hurtig and co-workers (Korhonen et al. 2002) reported that values of compressive stiffness and poisson's ratio of bovine cartilage derived from confined compression were slightly higher than values derived from unconfined compression tests, and values derived from indentation testing were significantly higher than both the confined and unconfined values. This technique dependence of mechanical properties is due to the inhomogeneous structure and anisotropic mechanical properties of cartilage.

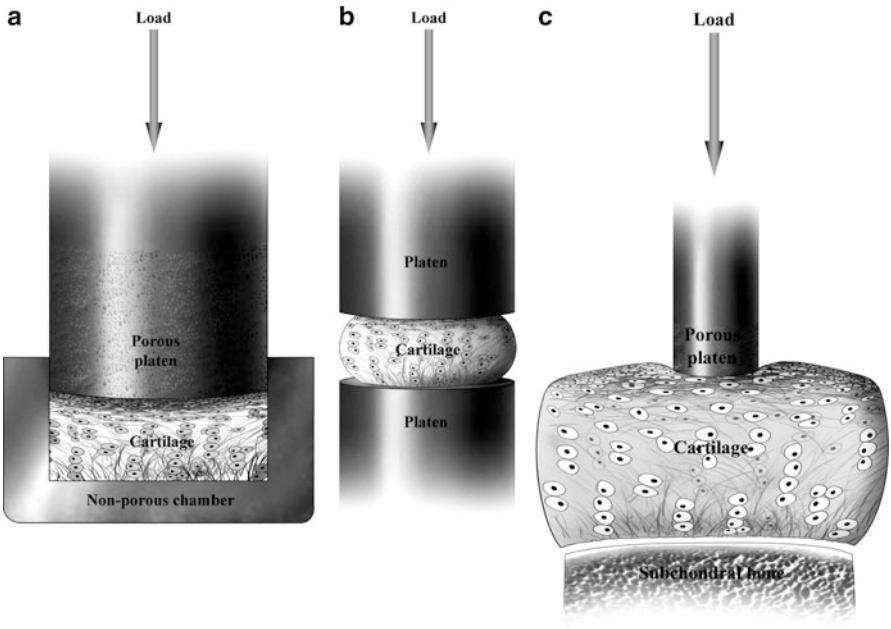


Fig. 41.3 Schematic representation of experimental setup used for the (**a**) confined, (**b**) unconfined compressive and (**c**) indentation testing of cartilage explants

41.1.4.5 *In Vivo* Mechanical Testing

The ability to monitor the health status of an intact cartilage, predict or diagnose osteoarthritis, and monitor the healing process of the tissue after a treatment had necessitated *in vivo* strategies, be it simple observation, or qualitative or quantitative measurements. Earlier techniques were based on magnetic resonance imaging (MRI). To this extent, Eckstein and co-workers (1999) analysed the deformation and recovery, as indicated by interstitial fluid flow rate in seven healthy patellae joints. Similarly, O'Byrne et al. (2003) assessed the biochemical composition of cartilage in goat knees, in response to papain injection. The authors were able to demonstrate a compromise of the tissues collagen integrity with the magnetic resonance technique. This dose-dependent degradation was confirmed by post-mortem biochemistry and histology.

High-frequency ultrasound and mechanical indentation in now commonly combined to measure both structural and mechanical parameters, such as stiffness, and thickness, respectively. In an example, Kiviranta et al. (2008) compared the dynamic stiffness of healthy and degenerated patella cartilage, thereby diagnosing early stages of OA. In a similar study, Nishitani et al. (2008) were able to arthroscopically determine the thickness and surface roughness of ten male athletes while undergoing mosaicplasty for steochondritis dissecans.

41.1.5 Modelling Theories for Articular Cartilage

During indentation tests, after a sudden application of constant load on cartilage, a rapid compression takes place, which is then followed by a slow creep process towards equilibrium at a rate which is governed by the applied load and test conditions (Mow et al. 1984). Early explanations for this viscoelastic behaviour did not take into account the interstitial fluid flow and internal redistribution of the organic matrix and the compaction within the cartilage specimen. Although the possible influence of the multiphasic nature of cartilage on its deformational characteristics was realised by Hirsch (1944) as early as 1944, the role of fluid flow on the dynamic deformational behaviour of articular cartilage was not recognised until later. One of these studies (Elmore et al. 1963) showed that the creep response observed in indentation testing of cartilage was largely due to the efflux of interstitial fluid from the tissue. They also observed that upon removal of the load, complete recovery of the tissue occurred only if sufficient fluid was available to re-imbibe into the tissue. Indeed, Linn and Sokoloff (1965) recorded a positive correlation between creep response and the amount of fluid exuded from cartilage tissue. Such studies stimulated a range of models describing both physicochemical and mechanical properties of cartilage.

41.1.5.1 Biphasic Theory

The biphasic model depicts cartilage as a soft, porous and permeable material comprising 20% (wt/vol) of elastic solid, and filled with an incompressible fluid (Mow et al. 1980; Torzilli and Mow 1976). The model accounts for the effect of the drag forces arising from the relative motion between the fluid and solid phases. It incorporates all existing known mechanical properties of cartilage, namely, inhomogeneity, anisotropy, stress-strain non-linearity, interstitial fluid flow and finite deformation. However, several assumptions are associated with the theory (Mow et al. 1992), namely,

- The solid matrix is porous, permeable, and elastic.
- The solid matrix and interstitial fluid are intrinsically incompressible; i.e. volume change of the tissue as a whole is possible only if there is fluid exudation or imbibition.
- Frictional drag is directly proportional to the relative velocity between the interstitial fluid and the porous-permeable solid matrix – the proportionality coefficient is the drag coefficient [K], which may be strain-dependent.
- The frictional drag of the interstitial fluid flow is the dominant mechanism controlling tissue viscoelasticity in compression.

A modified, general form is the Kuei, Lai and Mow biphasic theory for cartilage. This form differs to the previous by the addition of more constitutive assumptions, such as an infinitesimal strain, linear, isotropy, constant elastic coefficients, and

constant or strain-dependent permeability. However, some authors have associated the biphasic theory with inherent flaws (Brown and Singerman 1986). In particular; the theory relies on the ability to define the distinct phases, which is problematic as there are no distinctive barriers between the matrix and the fluid components. When applied to the prediction of creep behaviour of an isotropic, homogeneous and linearly elastic material undergoing small strain deformation the biphasic theory was found to be inadequate. For example, it was incapable of modelling the substantial portion of the transient phase of cartilage response when load under a slow rate in unconfirmed compression.

41.1.5.2 Triphasic Theory

It has been observed that when unloaded cartilage specimens are soaked in a sodium chloride (NaCl) solution at constant temperature, the tissue dimensions decrease exponentially with increasing NaCl concentration. The influence of ionic movements in cartilage on its swelling and deformational behaviour has long been recognised (Maroudas 1979). This has led to the development of the triphasic theory. The theory couples both the physicochemical aspects of cartilage swelling and the biphasic view of solid matrix deformation and interstitial fluid flow. The theory describes the equilibrium free swelling and confined compression behaviour of cartilage and other soft hydrated tissues. In this theory, cartilage is considered as a mixture of three phases: an *incompressible solid phase*, which is the matrix, consisting of collagen and PG, *an incompressible fluid phase*, which is the interstitial water, and an *ionic phase* of two species of a single salt, the cations and the anions. The theory can be applied to equilibrium as well as transient problems, and has been found capable of predicting the stress-strain fields in the solid matrix, the interstitial fluid flow along with the distribution of the ions, and fluid pressure (Gu et al. 1997; Lai et al. 1991).

41.1.5.3 Poroviscoelastic and Poroelastic Theories

Both the biphasic and triphasic theories fail to incorporate the anisotropy and viscoelasticity of cartilage, which are of great importance when determining cartilage mechanical properties. To this extent, several models exist, whose details are beyond the scope of the present review. More relevant is the consideration of the interfibrilar pores in cartilage, which control the transport of soluble nutrients and the flow-independent viscoelasticity of cartilage mechanical and physicochemical properties. These are described by the poroelastic and the poroviscoelastic theories.

The poroviscoelastic model describes the viscoelasticity exhibited by cartilage with a combination of a fluid flow-dependent, fluid flow-independent mechanisms and the intrinsic viscoelasticity of the solid matrix (Mak 1986). In the poroelastic model of cartilage, the tissue is modelled as an isotropic solid matrix containing fluid-saturated pores, entrapped by a fibrillar network. Both the solid and the fluid

phases are assumed to be incompressible. The structure is defined by the Young's modulus, Poisson's ratio of the matrix and the hydraulic permeability. The model assumes that the hydraulic permeability depends on the dilatation of the bulk material. The fibrils are evenly distributed in the radial, circumferential and axial directions forming an elastic constituent attached to the porous matrix and that the stiffness of the fibrillar network depends on the longitudinal strain of the fibrils. These fibrils have no resistance to compression and the effect of lateral deformation of every single fibril is neglected (Li et al. 1999).

41.1.6 Pathologies

41.1.6.1 Mechanical

Single and multiple blunt impacts on cartilage yielding 20% strains at strain rate of 6.7%.s^{-1} have been found to cause destruction of bovine metacarpal cartilage. Moreover, strains of 40% and above have evidently caused surface defects, correlating to collagen network failure and cell death. However, cartilage has been shown to survive impacts yielding less than 10% strain, with no injury to chondrocytes on their ECM (Radin et al. 1970; Repo and Finlay 1977). On the other hand, low impact may lead to cell death despite structural integrity being maintained (Duda et al. 2001).

Defects of articular cartilage may or may not reach the surface of the underlying bone. In care of the latter, these are termed chondral, or partial thickness defects. Some of these superficial lesions result from surgical procedures (Rosenberg 1971; Thompson 1975). Full thickness lesions, also termed osteochondral defects (Fig. 41.4), cross the tidemark of articular cartilage and violate the underlying subchondral bone. In doing so, they have access to cells in the bone marrow cavities.

41.1.6.2 Degenerative and Non-degenerative Diseases

Common diseases, which affect the health and functionality of the joint, are osteoarthritis (OA), rheumatoid arthritis (RA), chondromalacia and disuse atrophy. OA is a slowly progressive disorder of unknown cause (Mankin 1974a), which generally occurs later in life, principally affecting major weight-bearing joints. It is characterised clinically, by pain, deformity and reduced mobility, and pathologically, by features including focal erosive lesion, cartilage destruction, subchondral sclerosis, cyst formation, and large osteophytes at the margins of the joints. With the progression of OA, cartilage exhibits histological, biochemical and metabolic changes, although their precise nature frequently depends on the underlying abnormality and the duration of the disease progression. At the early stages of the disease, the tissue erodes, disappearing completely from the focal areas of the surface, leaving a denuded, sclerotic and eburnated bone. Type II collagen degrades beneath the articular surface, and their organisation is disrupted. Consequently, the tissue depletes in stiffness and strength and fibrillation follows (Mow et al. 1992).

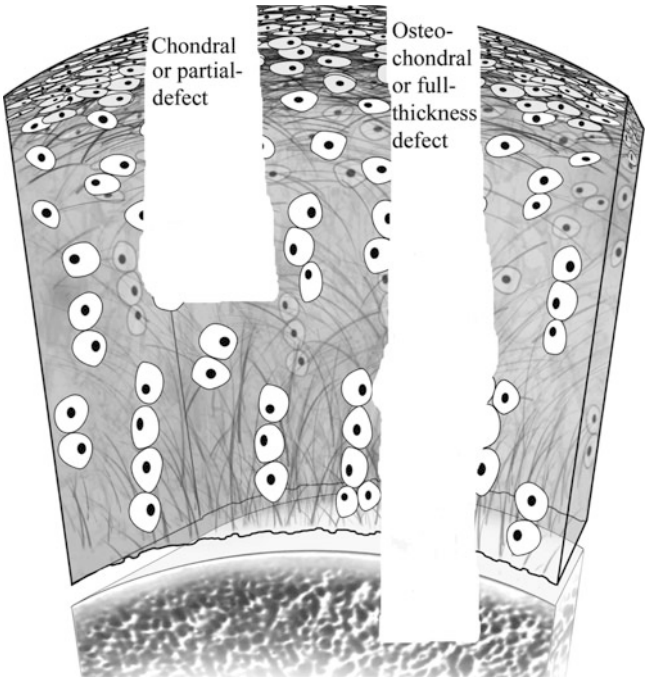


Fig. 41.4 Schematic representation of full and partial thickness defects in articular cartilage

RA typically affects many different joints and can be chronic in nature. This systemic disease affects the entire body and is one of the most common forms of arthritis. It is characterised by the inflammation of the membrane lining the joint, causing pain, stiffness, warmth, redness, and swelling. In a similar manner to OA, there is degradation of type II collagen, particularly around chondrocytes in the deep zones, rather than directly beneath the articular surfaces (Mow et al. 1992). PGs are also degraded, but can be partly replaced. Eventual cartilage thickness is reduced due to its exposure of migrating cytokines, produced in the adjacent subchondral bone, resulting in the erosion of underlying calcified cartilage and bone.

Pathological diseases of the articular cartilage are not necessarily confined to the elderly. Indeed, any form of joint immobilisation, for example following an injury or surgery, will lead to tissue atrophy and joint stiffness. These conditions can be reversed with joint remobilisation, starting with gentle exercise, which gradually increase in intensity. Another non-degenerative disease is chondromalacia patella, which is often caused by trauma, overuse, part misalignment or muscle weakness. Instead of gliding smoothly, the patella translates across the femur, thereby roughening the cartilage underneath the patella. The damage may range from a slight abnormality to a complete wear of the associated cartilage surface. Traumatic chondromalacia occurs when a blow to the patella bone tears off either a small piece of articular cartilage or a large fragment containing a piece of bone.

The latter is termed an osteochondral fracture. Clinically, the process results in mild to moderate pain and stiffness. The resulting changes resemble those of mild OA, with fibrillation, surface irregularities and cartilage erosion (Mankin 1974b).

41.2 Repair Strategies

41.2.1 Response to Injuries

The effects of mechanical injuries to articular cartilage vary considerably, depending on its nature and severity. Its response to superficial defects, which violate neither its calcified layers nor the underlying bone typically lack inflammation of the cartilage, and has limited potential for self-repair (Buckwalter 1998; Mankin 1982). Characteristically, there is minimal attempt on the part of the cartilage to elicit cellular or matrix repair (Calandruccio and Gilmer 1962; Campbell 1969; DePalma et al. 1966). However, cartilage responds to lacerative injuries with an enhanced mitotic activity adjacent to the defect margins. This is associated with increased synthesis of the matrix components (Mankin 1962).

When a cartilage defect affects the vasculature of the subchondral bone, an enhanced biological response is elicited. This repair response is equivalent to that of other vascularised tissues in the body (Mankin 1982), filling the whole defect cavity with blood. In the proceeding events, a blood clot is formed, which contains both red and white blood cells, undifferentiated cells and marrow elements (DePalma et al. 1966). However, only the bone defect is filled with the new bone, and is fused with the cartilage defect, with its edges united by the vascular fibrous tissue (Calandruccio and Gilmer 1962; DePalma et al. 1966). As observed with superficial defects, brief synthetic activities take place in the remaining cartilage, during which a small amount of cells and matrix is produced, replacing some of that lost to the initial damage (Mankin 1962, 1982).

The quality of the repaired cartilage is dependent on the initial defect size. For example, defects less than 3 mm in diameter often repair completely after 3 months and are difficult to locate after 9 months (Convery et al. 1972). However, defects 9 mm or larger may not completely repair. It has also been demonstrated that the site of an old osteochondral laceration may clearly be visible years after injury as a slightly discoloured, roughened pit, or linear grooves on the otherwise smooth surface adjacent to the defect site (Bennett and Bauer 1935; Campbell 1969; Key 1931).

41.2.2 Non-invasive Therapies

Different interventions exist for the management of cartilage damage. The most topical of these are lifestyle changes, pharmacological and surgical methods. The emphasis on lifestyle becomes highly relevant due to the high contributions of

obesity, and abnormal loading on the development and progression of osteoarthritis. Acute joint injuries, fractures of articular surface, along with tears of the meniscus and ligaments are all linked with osteoarthritis. Occupation and nutrition have also been deemed strong factors in degenerative cartilage diseases (Cooper et al. 1992; Felson and Zhang 1998; Lievense et al. 2001). For example, strong evidence exists, which suggests that the risk of OA doubles after 10 years of farming (Jensen 2008). Additionally, occupations which involve kneeling, squatting or heavy lifting also accelerates cartilage degeneration. Therefore, strategies such as weight control, recreational exercise, and injury prevention are all common interventions adopted as least-invasive therapies. More specific, exercises such as quadriceps strengthening; stretching and aerobic exercises are commonly prescribed for treatment of hip and knee OA (Bukowski et al. 2006; Roddy et al. 2005). Other examples are ultrasound (Soren 1965; Welch et al. 2001) and acupuncture (Brinkhaus et al. 2007; Lin and Chen 2009; Reinhold et al. 2008).

Pharmacological interventions play a vital role in pain relief to OA patients. For mild or moderate pain, acetaminophen (paracetamol) is a common choice by physicians (Towheed et al. 2006; Wegman et al. 2004). Other non-steroidal anti-inflammatory drugs (NSAID) such as naproxen and ibuprofen used for patients with either hip or knee OA have been found to be superior to paracetamol, in bringing pain relief, albeit, at a higher health risks. However, both analgestics have been associated with discomfort, perforation, and bleeding of the gastrointestinal tracts (Zhang et al. 2004). Other pharmacological interventions include chondroitin and glucosamine (Dahmer and Schiller 2008; Matsuno et al. 2009; Owens et al. 2004). While paracetamol and NSAIDs work by inhibiting the actions of cyclo.oxygenase-1 and -2, thereby, relieving the patients of pain, chondroitin and glucosamine are taken as supplements, which are used as building blocks during the ongoing turnover of cartilage.

41.2.3 Surgical Interventions

In cases where non-invasive therapies are not possible, due to substantial cartilage damage, or detachment of cartilage fragments, surgical procedures are undertaken. These include joint lavage, subchondral stimulation, autogenic and allogenic transplantation and cell transplantation, performed either arthroscopically or with an open joint surgical approach

41.2.3.1 Arthroscopic Cleaning

Arthroscopic procedures, such as lavage, involve thoroughly rinsing the joint cavity with Ringer solution, lactate and sodium chloride solutions. Such a palliative approach is designed to reduce the degree of the pain experienced by the patient. In the main, this strategy is successful, although the biological reasoning for pain relief

is not established (Anderson et al. 1993; Chang et al. 1993; Gillespie and O'Connell 1992; Livesley et al. 1991). However, there are limits to its application, particularly with OA patients whose pain relief is generally considered to be a result of a placebo effect (Gibson et al. 1992; Moseley et al. 1996).

Other arthroscopic strategies include chondral shaving and debridement. Chondral shaving involves the removal of the diseased tissue, while debridement combines chondral shaving with lavage to remove free bodies from the joint. As with lavage, the biological rationale behind each of these to procedures remains unclear. Indeed, cell loss along the lesion borders of the remaining cartilage has been reported to follow chondral shaving, which is counter-productive for cartilage repair (Kim et al. 1991; Mitchell and Shepard 1987; Tew et al. 2000). In addition, debridement has been reported to be associated with skeletal misalignment and shown clinically to exacerbate the osteoarthritic condition (Messner et al. 2000). Both procedures can be carried out using a gentle cutting instrument incorporating laser light at a specific wavelength. Although the laser is useful for welding and fusing the tissue, performing a chondral shaving or a debridement using laser offers little advantages over mechanical cutting method (Vangsness and Smith 1995). In both cases, the structural integrity of the cartilage matrix with pre-tensioned water content is damaged in addition to the degradation process.

41.2.3.2 Subchondral Stimulation

Typically for superficial defects, a common repair strategy involves surgically accessing the adjacent bone-marrow spaces along with the bone, adipose tissues and the vascular spaces. Examples of such procedures include abrasion chondroplasty, Pridie drilling and microfracture techniques. The three procedures are very similar, although the holes in the microstructure techniques are distributed across the entire cartilage lesion site, approximately 3 mm apart to a depth of 4 mm. Moreover, the holes are relatively small (0.5–1.0 mm diameter) when compared to Pridie drilling involving holes of between 2.0 and 2.5 mm in diameter.

By penetrating the subchondral bone beneath the defect, the void is immediately filled with a fibrin clot which, within 2 days adheres to the bony compartments of the wound, as opposed to the cartilaginous tissue. By the fifth day, mesenchymal stem cells have penetrated and completely resorbed the fibrin clot, filling the void. Thereafter, between days 10 and 14, the cells differentiate into chondrocytes and lay down a PG-rich matrix. By 8 weeks, the repair tissue begins to resemble normal cartilage and forms a continuous surface with the surrounding native tissue by approximately 6 months. However, by 12 months, there is generally evidence of degradation of repair tissue (Hunziker 1999; Wyre and Downes 2000). There is an inherent discontinuity between the repair tissue and the surrounding cartilage, as the collagen fibrils within the two compartments fail to integrate. Additionally, some PGs in the cartilage matrix exhibit anti-adhesive properties, and hinder the bonding between the repair and the native cartilage tissues. The employment of abrasion chondroplasty for cartilage defects in rabbits and dogs have resulted in the formation of cartilage-like tissue,

which originated from the subchondral bone (Altman et al. 1992; Kim et al. 1991), though other studies report the presence of significant quantities of fibrous cartilage (Furukawa et al. 1980). It has also been reported that the repaired full-thickness cartilage lesion in rabbits are more durable following the Pridie drilling when compared to the abrasion chondroplasty strategy (Menche et al. 1996). Indeed the Pridie approach has been reported to be of great benefit to patients with conditions such as osteochondritis dissecans and gonathrosis (Pedersen et al. 1995), yielding both pain relief and restored joint function (Beiser and Kanat 1990; Goldman et al. 1997). The microfracture technique, being a minimally invasive arthroscopic procedure is relatively less disruptive to the subchondral bone. Nonetheless, its employment in treating young athletes and horses has resulted in an improved joint function and pain relief (Frisbie et al. 1999; Sledge 2001).

41.2.3.3 Tissue Grafting

The transplantation of cartilage into defect sites has been a viable strategy for several decades (Cohen and Lacroix 1955). The transplanted cartilage may be sourced autologously or extracted from cadavers. In autologous cartilage transplantation, plugs of cartilage biopsy are extracted either from adjacent to the defect (periosteal) or from the rib (perichondral). These are either sutured or glued to the defect floor, such that the defect may be stimulated to form repair cartilage that binds to the transplant, forming a continuous neo-tissue over the entire defect (Ohlsen 1976). With this technique, joint function and pain relieve have been reported to reach 80% of cases (Bouwmeester et al. 1997; Homminga et al. 1990; Korkala and Kuokkanen 1991; Moran et al. 1992). Moreover, these strategies are advantageous because they minimise disease transfer and immunological rejection, which are commonplace with allografts from another donor. For these reasons, autografts have produced treatments with survival rates up to 70% at 2–5 years (Temenoff and Mikos 2000). In general, chondrogenesis associated with periosteal grafts is relatively superior, as perichondral grafts often fail due to ossification (Nehrer et al. 1999). In addition, explants taken from non-load-bearing region of cartilage is often unable to withstand forces imparted at joint surfaces. Due to its lack of mechanical integrity, the matrix of the implant and cartilage in the vicinity of the defect breaks down to the extent that the implant and associated regions later exhibit signs of osteoarthritis. Additionally, this breakdown also occurs at the donor site (Kim et al. 1991; Mitchell and Shepard 1987), often necessitating undesirable and expensive second operation. Indeed, the amount of autograft that can be harvested is limited, thus the technique is often unsuitable for clinical-sized defects.

Alternately, allogenic osteochondral grafts obtained from human cadavers have been used to fill cartilage defects. Unlike autologous grafts, no biological interaction is predicted between the transplant and its surrounding cartilage, such that the primary role of the transplant is to fill the defect site and replace the lost tissue volume. This approach has benefited patients with large osteochondral defects, particularly caused by trauma and osteo-necrosis. The benefits of osteochondral resurfacing in

the human knee joint has been observed to last for many years (Bakay et al. 1998; Bell et al. 1994), with reported success rates such as 85% after 10 years (Mahomed et al. 1992; Meyers et al. 1989). Although, adverse immunological reactions are associated with this procedure, the survival of allogenic transplants may be prolonged with the use of immunosuppression and histocompatibility techniques (Hickey et al. 1994; Stevenson 1987; Stevenson et al. 1989).

The perichondrium, which is a dense membrane composed of fibrous connective tissue that closely wraps cartilage (except for the articular cartilage, which is covered by the synovial membrane) has been implanted unto cartilage defects in the human joints (Homminga et al. 1990; Kwan et al. 1989). These autologous scaffolds were advantageous because they naturally contain autogeneous cells that are useful for cartilage repair. However, in addition to the limited availability of harvest sites, the neo-cartilage formation resulting from these grafts have been deemed unsatisfactory in patients over the age of 40 (Seradge et al. 1984). Moreover, regenerates formed from these scaffolds do not completely fill the defect and tend to detach and ossify (Hendrickson et al. 1994; Homminga et al. 1990). Another autologous scaffold is the ostechondral autograft. These are biopsies from osteochondral plugs removed from low-weight-bearing areas on the femoral condyles. Similarly to the perichondrial grafts, the osteochondral plugs are populated with the patients-own cells. However, the neo-cartilage formed does not survive indefinitely. This is partly due to the damaged sustained during their implantation procedure, which involves hammering into the defect site (Laprell and Petersen 2001). Moreover, defects are created at the sites where the plugs are biopsied, though non-load-bearing; these may lead to future complications (Hurtig et al. 2001).

41.2.3.4 Cell Transplantation

Isolated chondrocytes have been transplanted into articular cartilage defects for its repair. However, such an approach typically has a success rate of less than 40%, as the cells are not retained within the defect site for sufficient period to produce neo-ECM (Temenoff and Mikos 2000). On the other hand, mesenchymal stem cells from the skeletal muscle of adult rabbits, seeded onto porous polyglycolic acid (PGA) mats have also been implanted into non-weight bearing defects in the rabbits femoropatella groove. The PGA matrix biodegrades and the stem cells remain *in situ*, producing a cartilage-like tissue containing type II collagen and subchondral bone that is morphologically similar to native tissue (Grande et al. 1997; Martin et al. 1999). However, in a similar approach using mesenchymal stem cells and collagen gel for cartilage defects in osteoarthritic human knees limited clinical improvement was observed after 42 weeks (Wakitani et al. 2002).

41.2.3.5 Autologous Cell Implantation (ACI)

Since its first clinical application for treating deep articular cartilage defects in the knee (Brittberg et al. 1994), ACI has been a fairly successful approach for treating

cartilage defects. The technique involves harvesting a healthy or non-healthy (Dehne et al. 2009) portion of cartilage, usually from a non-load bearing region of the patient, and enzymatically degrading the tissue to isolate the cell population. These cells are expanded *in vitro* to a sufficient density for implantation, approximately of the order of 50×10^6 cells.ml^{-1}, into the defect site which had been cleared and prepared using a purposely designed curette. The cells used in the repair may either originate from the chondrocytes in the host-extracted cartilage, its precursor cells from the periosteum or possibly mesenchymal stem cells of the subchondral bone (of the defect site) had this been injured. The use of ACI has resulted in an improved joint function for at least 72% of patients at 1 year post-operatively (Bentley et al. 2003; Minas 1998) and 84% of patients at 3 years post-operatively (Micheli et al. 2001). For instance, ACI has been used for treating defects in other joints such as hips and ankles (Giannini et al. 2001; Romeo et al. 2002). The use of ACI as an alternative treatment to surgical excursion, allogenic grafting and autografting (Peterson et al. 2003) has been discussed to provide long-term joint restoration and pain relief for patients with osteochondritis dissecans.

41.2.4 Tissue Engineering

Despite the numerous strategies available for treating cartilage defects, there is yet to be a standardised solution for restoring long term function, especially due to the large variability of defects to be treated. This limitation has encouraged a more sophisticated tissue-engineered approach (Ringe and Sittinger 2009). Tissue engineering combines the principles of cell and molecular biology with material technology, to create a new tissue, which has the potential to physically and biologically mimic its predecessor and restore function to the damaged tissue. Key activities in this approach are the attainment and expansion of cells, the development of scaffolds that act as carriers for the cells.

41.2.4.1 Chondrocyte Source

An important step in tissue engineering is the isolation and expansion of cells that are to be transplanted. The cells must be both appropriate for the intended tissue and of sufficient quantity to treat clinical-sized defects (LeBaron and Athanasiou 2000), whilst being free of pathogens and contamination. Cells sources can be either autologous, allogenic or xenogenic, the latter being derived from a different animal species. Each approach has specific benefits and shortcomings (Breinan et al. 2001; Ma et al. 2005; Masuoka et al. 2005; Ostrander et al. 2001; Pavesio et al. 2003). For example, although autologous cells are free from imuno-related problems, without causing harvesting complications, they are relatively few in numbers. Thus the autologous approach does not effectively lead to a readily available off-the-shelf solution.

Allogenic and xenogenic cells may be extractible in large numbers and are available off-the-shelf, but these are associated with immunological problems and in the case of xenogenic cells, there is often the possibility of animal virus transmission (Sirlin et al. 2001). Tissue-engineered constructs derived from these cells require additional steps to incorporate immune acceptance.

41.2.4.2 Stem Cells

More recently, stem cells have been proposed to be a vital source of cells for tissue engineering applications. In a similar manner to differentiated cells, stem cells may either be autologous, allogenic or xenogenic in nature. Stem cells offer the benefits of being able to be multiplied extensively, yielding high cell number; from which, the desired number may be extracted and differentiated into chondrocytes. The remaining stem cells may be further multiplied for future use. As an example, mesenchymal stem cells derived either from the bone marrow and other adult connective tissues (Friedenstein et al. 1976) may differentiate to a selected range of cells including chondrocytes, osteoblasts, tenocytes or myocytes, irrespective of their origin (Jones et al. 2002; Minguell et al. 2001; Pittenger et al. 1999; Yoo et al. 1998). For the same reasons, embryonic stem cells, derived from the inner cell mass of the embryonic blastocyst; offer even great potentials for tissue engineering. Stem cells have the ability to multiply extensively before showing signs of senescence (von der Mark et al. 1977). However, there are ethical and legal concerns with using human embryonic cells. For this reason, much of the research has been conducted on animals (Fuchs et al. 2005; Kramer et al. 2006). Though instructive, fundamental differences between different species necessitate that those current findings resulting from animal models be confirmed on humans, before the therapeutic potential of embryonic stem cells are to be employed in tissue engineering and regenerative medicine.

41.2.4.3 Scaffold Technology

Cells and growth factors are commonly transplanted into the body with the support of a carrier scaffold. These carriers function to retain the cells at the defect site, allow them to multiply and synthesize their own ECM. Therefore, such scaffolds must provide a number of design properties including: (1) Biocompatibility: To prevent undesirable immune or biological response. (2) Permeability: to demonstrate sufficient porosity to enable good nutrient supply to cells at all regions of the construct, allow transport of signalling molecules between cells, permit removal of waste products and allow ingrowth of host tissue. (3) Biodegradability, where appropriate, to enable the scaffold to degrade in a controlled temporal manner, into non-toxic by-products as a neo-tissue is developed In addition, this property may enable the controlled release of morphogens and/or pharmacological agents to encourage cellular activity.

To date, a wide range of natural and synthetic materials are available for use as scaffolds for tissue engineered cartilage constructs (Barnewitz et al. 2006; Ossendorf et al. 2007; Perka et al. 2000; Risbud and Sittinger 2002; Sittinger et al. 1994, 2004). For example, the potential of fibrin based scaffolds as carriers of cells and growth factors for cartilage regeneration was investigated (Hendrickson et al. 1994). Although this naturally clot-forming polymer produces neo-tissue that is histologically similar to natural cartilage, it has poor mechanical properties and often evokes an immune response (Kawabe and Yoshinao 1991). The use of collagen-based scaffold for delivering cell and growth factors to defect sites is extensive. As collagen naturally occurs in skeletal tissues, it promotes attachment of cells unto its surface. Accordingly, it has been used either cell-free, seeded with chondrocytes or mesenchymal stem cells, in many animal studies (Russlies et al. 2002; Sams et al. 1995; Samuel et al. 2002). Chondrocytes seeded onto dense collagen scaffolds, implanted into rabbit femoral trochleas for up to 24 weeks was demonstrated to have produced a hyaline-like cartilage that was biochemically and mechanically similar to its surrounding cartilage (Frenkel et al. 1997). By contrast, other *in vivo* studies reported that although the repair appears adequate at earlier time point, subsequent thinning of the repair tissue occurs with time (Wakitani et al. 1994). Hyluronan is a non-sulphated GAG that is essential for the aggregation of large proteoglycans such as Aggrecans in articular cartilage. It has been used to deliver mesenchymal stem cells to caprine chondral defects (Butnariu-Ephrat et al. 1996), and stabilise chondrocytes and osteochondral progenitor cells for cartilage defects in rabbits (Grigolo et al. 2001; Solchaga et al. 2002). Although the newly developed tissues exhibit good integration with the host cartilage, they are typically thinner and often induce the breakdown of cartilage matrix.

Chitosans have been used to deliver cells and growth factors to the defect site. Chitosan, which can form into thermo reversible hydrogels, offers the combined advantages of an implant with an uniform distribution of cells and direct injectability into the defect. At lower temperature, chitosan is molten and is mixed with cell suspension. When injected into the body at 37 °C, the cell-gel solution solidifies into the defect (Chenite et al. 2000). However, their inferior mechanical property of the chitosan implant limits its use in a load-bearing environment.

Dissemination of chondrocytes within agarose and alginate hydrogels is a well established protocol for *in vitro* cartilage models (Benya et al. 1988; Freeman et al. 1994; Lee and Bader 1995). These systems have demonstrated their value in studying the response of chondrocytes to many external stimuli while excluding the coupled influences of other factors that are also implicated within the native cartilage. Examples of such studies include the effects of dynamic mechanical stimulation on chondrocyte metabolism (Chowdhury et al. 2001, 2003; Lee and Bader 1997) and chondrocyte deformation (Buschmann et al. 1995; Knight et al. 1998). In a similar manner to many hydrogels, however, cell-seeded agarose or alginate constructs are limited by their poor resorption rate and their inferior mechanical and biochemical properties, making them unsuitable for load-bearing applications.

Compared to natural scaffolds, the mechanical and biochemical properties of synthetic scaffolds are readily modified to suit specific applications. For cartilage regeneration, these are commonly derivatives of polyglycolic acids (PGA) and polylactic acids (PLA), both of which have been demonstrated to support chondrogenesis (Haisch et al. 2005). However, PGA was found to be weaker than most synthetic scaffolds, and degrades very fast, often releasing acidic byproducts of degradation into its immediate environment, which may prove cytotoxic (Grande et al. 2003). The uses of PLA/PGA copolymer as scaffolds allow improved control of the degradation rate. Indeed success was demonstrated by Cohen et al., who observed good histological and biochemical response, after 12-week implantation of the co-polymer into rabbit-chondral-defects (Cohen et al. 2003). An exhaustive range of copolymers have been proposed for cartilage repair. These include PLA/PEG (Polyethylene glycol) (Tamai et al. 2005), and nanofibrous forms of PLA/PCL (Polycaprolactone) (Li et al. 2005), the latter has been shown to elicit differentiation of human mesenchymal stem cells into chondrocytes exhibiting similar zonal morphology to that of which in native cartilage. Other synthetic copolymers include Polyethylene glycol-terephthalate/polybutylene-terephthalate (PET/PBT) (Weisser et al. 2001). Scaffolds derived from carbon fibres have also been used in cartilage lesions with good results (Mooney et al. 1996). Multiphase implants consisting of PGA, Bioglass and calcium phosphates have been examined in osteochondral defects in goats (Niederauer et al. 2000). The PGA fibres were seeded with autologous chondrocytes and the calcium phosphates were used to modulate the constructs stiffness at specific regions of the implants.

41.3 The Limiting Factor: What Lies Beneath

41.3.1 Subchondral Considerations in Cartilage Disease

Although little is known about the relationship between bone and cartilage in the etiology of osteoarthritis, an abnormal growth of the subchondral bone resulting in thickened subchondral bone plate, increased stiffness, and bone mineral density have been tagged with the progression of the disease (Fazzalari and Parkinson 1997; Grynpas et al. 1991; Li and Aspden 1997). Having observed these, and a decreased energy absorbing capacity Radin and co-workers proposed that stiffening of the subchondral plate was an initiating factor in osteoarthritis (Radin et al. 1970). They later hypothesised that trabecular microfracture due to impulsive loading initiates bone remodelling in the subchondral plate. This leads to localized stiffening that in turn produces increased shear stress in the cartilage, culminating in cartilage breakdown (Pugh et al. 1974)

It had been an ongoing debate that the calcified cartilage layer and the tide mark was an impenetrable structure, separating the articular cartilage from its underlying subchondral bone. However, microcracks and micro channels between the subchondral region and the uncalcified cartilage have been demonstrated

(Clark and Huber 1990; Holmdahl and Ingelmark 1950). It is therefore conceivable that these microcracks, and the vascularization in the subchondral bone plate, could facilitate molecular transport from the subchondral region to the basal layer of cartilage. Evidence to support this transportation comes from the discovery of hepatocyte growth factor (HGF) within the deep zone of normal cartilage, and an elevated level in osteoarthritic cartilage (Pfander et al. 1999); despite it not being produced by chondrocytes, but by the osteoblasts in the subchondral region (Guevremont et al. 2003). It has therefore been proposed that, following its synthesis by subchondral osteoblasts, HGF can reach the deep layers of articular cartilage via these microcracks, and/or the vascularised subchondral plate, and promote cartilage breakdown and/or enhances matrix remodelling. The association of HGF with Osteoarthritis comes from its incitement of MMP-13 production (Reboul et al. 2001); an enzyme present in the lower intermediate and deep layers of osteoarthritic cartilage. Other evidence for the role of subchondral bone in cartilage degradation are TGF-ß, Cathepsin K, and PGE2/LTB4, which are all produced by osteoarthritic subchondral bone cells, and yet found at the deep layers of osteoarthritic cartilage (Konttinen et al. 2002; Moldovan et al. 1997; Nakase et al. 2000).

41.3.2 Subchondral Considerations in Cartilage Healing

Although clinical studies have reported favorable results with osteochondral autografts at short and mid-term follow-up, animal studies 3 months after grafting found signs of degeneration, evidenced by chondrocyte clustering and hyper-cellularity in cartilage (Tibesku et al. 2004). Answering the question of whether the observed degradation may be detected histologically, Kleemann et al. (2007) characterized the mechanical competence and morphology of cartilage in osteochondral autografts. The ensuing study demonstrated that grafted tissues seem to undergo in a short period of time, where both substantial degenerative and regenerative processes occur. The compressive stiffness of the grafted cartilage was about 58% of that of healthy tissue at 3 months, and rose to 82% at 6 months. Fibrillation, hypercellularity, and cell clustering were observed at the edges of the grafts. Both the cartilage and the underlying bone were observed to be degrading, raising doubts as to their long term repair. Events such as disrupted nutrition (Malinin and Ouellette 2000), physical damage during the extraction, and transplantation of the graft (Buckwalter and Mankin 1998; Duda et al. 2001; Huntley et al. 2005; Redman et al. 2004; Whiteside et al. 2005) were thought to be contributing factors. Despite this, the intensity of type II collagen staining of the healthy and the grafted cartilage tissue were identical.

A bottom-upwards approach by Schell et al., had aimed to first support the reconstruction of the subchondral bone plate in an osteochondral defect, and thereby improving the mechanical and histological quality of the repaired cartilage

(Schell et al. 2007). The authors transplanted crushed bone graft together with a collagen membrane into osteochondral defects, 8.3 mm in diameter and 10 mm in depth. Comparing its healing with unfilled control groups, they observed no difference in healing outcome between the two groups after 6 months. All defects, whether filled or not, showed an irregular, more or less advanced cartilage repair. However, the articular surface was not restored in any case

A similar endeavour had attempted to encourage osteochondral healing through mechanical straining (Duda et al. 2005). Bone resorption and formation were observed at the base, and at the circumference of the defects, respectively. Defect filling, cartilage formation, and trabecular structures were observed for up to 12 weeks. Although their defects were completely filled, the neo-tissue mainly comprised of fibrous cartilage, and only partially with hyaline-like cartilage.

The importance of the subchondral bone in cartilage healing is undisputed. When a cartilage lesion is deep enough, the penetrated subchondral bone is prompted into action. Often, in cases such as abrasion chondroplasty and microfracture, it is strategically penetrated surgically for its input into the healing process to be realised. It is now a topical discussion that the state of the underlying bone itself, be it mechanical, physiological, or otherwise, is actually important for the quality of cartilage regenerated.

41.3.3 Subchondral Considerations in Cartilage Tissue Engineering

The functioning of articular cartilage is believed to be dependent on the mechanical support by the subchondral bone. In fact, the steep stiffness gradient in the subchondral bone is suggested to be responsible for the initiation and progression of cartilage damage (Radin and Rose 1986). Moreover, the stiffened subchondral bone associated with osteoarthritis (Radin et al. 1970) is said to cause transverse stresses at the base of the articular cartilage, potentially resulting in deep horizontal splits therein. Given their apparent differences, tissue-engineered solutions either favours cartilage repair, or bone regeneration, and seldom satisfy both tissues. Osteochondral repair strategies now aim to concurrently mimic the physiological properties and structure of the cartilage and bone using cell-seeded constructs. The resulting hybrid is an engineered scaffold consisting of both a cartilage-optimised phase and a subchondral bone-optimised phase (Temenoff and Mikos 2000). Principally, the two phases are produced separately, under their appropriate conditions, and with the appropriate cellular disseminations, and are later united prior to implantation. On this front, two kinds of hybrid scaffolds have been developed by Hutmacher and co-workers, using a combination of fibrin, polycaprolactone (PCL), and a PCL-TCP combination. In one instance, the fibrin served as the cartilage phase while the PCL scaffold substitutes for the subchondral phase. On another occasion, their hybrid consisted of PCL and PCL-TCP. The top PCL region promotes cartilage regeneration

while the underlying PCL-TCP serves as the subchondral bone phase. Having been seeded with pre-cultured MSCs, the biphasic constructs were implanted into New Zealand white rabbits for up to 6 months. The researchers found that in terms of cartilage regeneration, PCL bettered the fibrin constructs; stipulating that the mechanical support provided by the fibrin was insufficient for cellular development, and its subsequent secretion of the essential ECM products. In fact, the fibrin degrades rapidly, while the porous PCL scaffold degraded slowly, providing an effective mechanical support.

Along a similar line, Schlichting et al. evaluated the healing of osteochondral defects using polylactideco-glycolide scaffolds of differing stiffness, hypothesizing that a stiff scaffold creates sufficiently stable conditions necessary for subchondral bone formation and consequently cartilage regeneration compared with a softer scaffold or to untreated controls (Schlichting et al. 2008). The stiff scaffold was found to improve the regeneration of subchondral bone, while the soft scaffolds provided less support, and consequently the surrounding subchondral bone became more sclerotic. Indeed, the regenerated cartilage that was formed over, the stiff scaffold exhibited higher elastic and dynamic moduli at 3 months than did the soft scaffold group. However these mechanical properties were not dissimilar for both groups at 6 months. Moreover, these values were inferior to that of native articular cartilage. These findings led to the conclusions that materials used to fill subchondral defects should have a comparable stiffness to that of healthy subchondral bone rather than being too flexible. When this is not the case, degradation or resorption of filling materials will lead to loss of stiffness, and may compromise the defect healing.

41.4 Summary

The present review has looked at the current challenges faced when trying to regenerate cartilage. Most of these issues have been related to the structure, composition, and mechanical features of the tissue. In light of the topics discussed above, the following statements may summarise the current challenges associated with cartilage regeneration:

Cartilage is a complex tissue, with at least three phases. Moreover, cartilage illness may be systemic, local, acute or chronic. Current treatment options aim to reduce pain. By large, there is as of yet no solution that is all-encompassing, and can regenerate all the different types of cartilage defects. Despite the ongoing debate over the separation of articular cartilage from its subchondral bone by the tide mark, there exists an overwhelming amount of evidence to link the two regions, particularly at the onset of OA. Therefore, a good strategy for cartilage regeneration ought not to neglect the underlying subchondral tissue. Principally, a successful clinical outcome will have re-established both the damaged cartilage and its underlying subchondral bone.

References

Altman RD, Kates J, Chun LE, Dean DD, Eyre D (1992) Preliminary observations of chondral abrasion in a canine model. Ann Rheum Dis 51:1056–1062

Anderson MA, Payne JT, Kreeger JM, Wagner-Mann CC, Schmidt DA, Mann FA (1993) Effects of intra-articular chlorhexidine diacetate lavage on the stifle in healthy dogs. Am J Vet Res 54:1784–1789

Ateshian GA, Wang H (1997) Rolling resistance of articular cartilage due to interstitial fluid flow. Proc Inst Mec Eng 211:419–424

Ayad S, Kwan AP, Grant ME (1987) Partial characterization of type X collagen from bovine growth-plate cartilage. Evidence that type X collagen is processed in vivo. FEBS Lett 220:181–186

Bakay A, Csonge L, Papp G, Fekete L (1998) Osteochondral resurfacing of the knee joint with allograft. Clinical analysis of 33 cases. Int Orthop 22:277–281

Barnewitz D, Endres M, Kruger I, Becker A, Zimmermann J, Wilke I, Ringe J, Sittinger M, Kaps C (2006) Treatment of articular cartilage defects in horses with polymer-based cartilage tissue engineering grafts. Biomaterials 27:2882–2889

Beiser IH, Kanat IO (1990) Subchondral bone drilling: a treatment for cartilage defects. J Foot Surg 29:595–601

Bell RS, Davis A, Allan DG, Langer F, Czitrom AA, Gross AE (1994) Fresh osteochondral allografts for advanced giant cell tumors at the knee. J Arthroplasty 9:603–609

Bennett GA, Bauer W (1935) Further studies concerning the repair of articular cartilage in dog joints. J Bone Joint Surg Am 17:141–150

Bentley G, Biant LC, Carrington RW, Akmal M, Goldberg A, Williams AM, Skinner JA, Pringle J (2003) A prospective, randomised comparison of autologous chondrocyte implantation versus mosaicplasty for osteochondral defects in the knee. J Bone Joint Surg Br 85:223–230

Benya PD, Brown PD, Padilla SR (1988) Microfilament modification by dihydrocytochalasin B causes retinoic acid-modulated chondrocytes to reexpress the differentiated collagen phenotype without a change in shape. J Cell Biol 106:161–170

Boot-Handford RP, Tuckwell DS, Plumb DA, Rock CF, Poulsom R (2003) A novel and highly conserved collagen (pro(alpha)1(XXVII)) with a unique expression pattern and unusual molecular characteristics establishes a new clade within the vertebrate fibrillar collagen family. J Biol Chem 278:31067–31077

Bouwmeester SJ, Beckers JM, Kuijer R, van der Linden AJ, Bulstra SK (1997) Long-term results of rib perichondrial grafts for repair of cartilage defects in the human knee. Int Orthop 21:313–317

Breinan HA, Minas T, Hsu HP, Nehrer S, Shortkroff S, Spector M (2001) Autologous chondrocyte implantation in a canine model: change in composition of reparative tissue with time. J Orthop Res 19:482–492

Brinkhaus B, Witt CM, Jena S, Linde K, Streng A, Hummelsberger J, Irnich D, Hammes M, Pach D, Melchart D et al (2007) Physician and treatment characteristics in a randomised multicentre trial of acupuncture in patients with osteoarthritis of the knee. Complement Ther Med 15:180–189

Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 331:889–895

Brown TD, Singerman RJ (1986) Experimental determination of the linear biphasic constitutive coefficients of human fetal proximal femoral chondroepiphysis. J Biomech 19:597–605

Buckwalter JA (1998) Articular cartilage: injuries and potential for healing. J Orthop Sports Phys Ther 28:192–202

Buckwalter JA, Mankin HJ (1997) Articular cartilage. Part I: tissue design and chondrocyte-matrix interactions. J Bone Joint Surg Am 79A:600–611

Buckwalter JA, Mankin HJ (1998) Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. Instr Course Lect 47:487–504

Buckwalter JA, Rosenberg LC, Ungar R (1987) Changes in proteoglycan aggregates during cartilage mineralization. Calcif Tissue Int 41:228–236
Buckwalter JA, Mankin HJ, Grodzinsky AJ (2005) Articular cartilage and osteoarthritis. Instr Course Lect 54:465–480
Bukowski EL, Conway A, Glentz LA, Kurland K, Galantino ML (2006) The effect of iyengar yoga and strengthening exercises for people living with osteoarthritis of the knee: a case series. Int Q Community Health Educ 26:287–305
Buschmann MD, Gluzband YA, Grodzinsky AJ, Hunziker EB (1995) Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. J Cell Sci 108(Pt 4):1497–1508
Butnariu-Ephrat M, Robinson D, Mendes DG, Halperin N, Nevo Z (1996) Resurfacing of goat articular cartilage by chondrocytes derived from bone marrow. Clin Orthop Relat Res 330:234–243
Calandruccio RA, Gilmer WSJR (1962) Proliferation, regeneration, and repair of articular cartilage of immature animals. J Bone Joint Surg Am 44:431–455
Campbell CJ (1969) The healing of cartilage defects. Clin Orthop Relat Res 64:45–63
Chang RW, Falconer J, Stulberg SD, Arnold WJ, Manheim LM, Dyer AR (1993) A randomized, controlled trial of arthroscopic surgery versus closed-needle joint lavage for patients with osteoarthritis of the knee. Arthritis Rheum 36:289–296
Cheifetz S, Bassols A, Stanley K, Ohta M, Greenberger J, Massague J (1988) Heterodimeric transforming growth factor beta. Biological properties and interaction with three types of cell surface receptors. J Biol Chem 263:10783–10789
Chenite A, Chaput C, Wang D, Combes C, Buschmann MD, Hoemann CD, Leroux JC, Atkinson BL, Binette F, Selmani A (2000) Novel injectable neutral solutions of chitosan form biodegradable gels in situ. Biomaterials 21:2155–2161
Chowdhury TT, Bader DL, Lee DA (2001) Dynamic compression inhibits the synthesis of nitric oxide and PGE(2) by IL-1beta-stimulated chondrocytes cultured in agarose constructs. Biochem Biophys Res Commun 285:1168–1174
Chowdhury TT, Bader DL, Shelton JC, Lee DA (2003) Temporal regulation of chondrocyte metabolism in agarose constructs subjected to dynamic compression. Arch Biochem Biophys 417:105–111
Clark JM, Huber JD (1990) The structure of the human subchondral plate. J Bone Joint Surg Br 72:866–873
Clarke IC (1971) Articular cartilage: a review and scanning electron microscope study. 1. The interterritorial fibrillar architecture. J Bone Joint Surg Br 53:732–750
Cohen J, Lacroix P (1955) Bone and cartilage formation by periosteum; assay of experimental autogenous grafts. J Bone Joint Surg Am 37-A:717–730
Cohen SB, Meirisch CM, Wilson HA, Diduch DR (2003) The use of absorbable co-polymer pads with alginate and cells for articular cartilage repair in rabbits. Biomaterials 24:2653–2660
Comper WD (1996) Extracellular matrix. Harwood Academic Publishers, Amsterdam
Convery FR, Akeson WH, Keown GH (1972) The repair of large osteochondral defects. An experimental study in horses. Clin Orthop Relat Res 82:253–262
Cooper C, Cushnaghan J, Kirwan JR, Dieppe PA, Rogers J, McAlindon T, McCrae F (1992) Radiographic assessment of the knee joint in osteoarthritis. Ann Rheum Dis 51:80–82
Dahmer S, Schiller RM (2008) Glucosamine. Am Fam Physician 78:471–476
Dehne T, Karlsson C, Ringe J, Sittinger M, Lindahl A (2009) Chondrogenic differentiation potential of osteoarthritic chondrocytes and their possible use in matrix-associated autologous chondrocyte transplantation. Arthritis Res Ther 11:R133
DePalma AF, McKeever CD, Subin DK (1966) Process of repair of articular cartilage demonstrated by histology and autoradiography with tritiated thymidine. Clin Orthop Relat Res 48:229–242
Duda GN, Eilers M, Loh L, Hoffman JE, Kaab M, Schaser K (2001) Chondrocyte death precedes structural damage in blunt impact trauma. Clin Orthop Relat Res 393:302–309
Duda GN, Maldonado ZM, Klein P, Heller MO, Burns J, Bail H (2005) On the influence of mechanical conditions in osteochondral defect healing. J Biomech 38:843–851

Eckstein F, Tieschky M, Faber S, Englmeier KH, Reiser M (1999) Functional analysis of articular cartilage deformation, recovery, and fluid flow following dynamic exercise in vivo. Anat Embryol 200:419–424

Eckstein F, Lemberger B, Stammberger T, Englmeier KH, Reiser M (2000) Patellar cartilage deformation in vivo after static versus dynamic loading. J Biomech 33:819–825

Elmore SM, Sokoloff L, Norris G, Carmeci P (1963) Nature of "imperfect" elasticity of articular cartilage. J Appl Physiol 18:393–396

Eyre DR (2004) Collagens and cartilage matrix homeostasis. Clin Orthop Relat Res 427:S118–S122

Eyre DR, Jiann-Jiu W, Woods P (1992) Cartilage-specific collagens, structural studies. In: Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds) Articular cartilage and osteoarthritis. Ravens Press Ltd., New York

Fazzalari NL, Parkinson IH (1997) Fractal properties of subchondral cancellous bone in severe osteoarthritis of the hip. J Bone Miner Res 12:632–640

Felson DT, Zhang Y (1998) An update on the epidemiology of knee and hip osteoarthritis with a view to prevention. Arthritis Rheum 41:1343–1355

Freeman PM, Natarajan RN, Kimura JH, Andriacchi TP (1994) Chondrocyte cells respond mechanically to compressive loads. J Orthop Res 12:311–320

Frenkel SR, Toolan B, Menche D, Pitman MI, Pachence JM (1997) Chondrocyte transplantation using a collagen bilayer matrix for cartilage repair. J Bone Joint Surg Br 79:831–836

Friedenstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267–274

Frisbie DD, Trotter GW, Powers BE, Rodkey WG, Steadman JR, Howard RD, Park RD, McIlwraith CW (1999) Arthroscopic subchondral bone plate microfracture technique augments healing of large chondral defects in the radial carpal bone and medial femoral condyle of horses. Vet Surg 28:242–255

Fuchs JR, Hannouche D, Terada S, Zand S, Vacanti JP, Fauza DO (2005) Cartilage engineering from ovine umbilical cord blood mesenchymal progenitor cells. Stem Cells 23:958–964

Furukawa T, Eyre DR, Koide S, Glimcher MJ (1980) Biochemical studies on repair cartilage resurfacing experimental defects in the rabbit knee. J Bone Joint Surg Am 62:79–89

Giannini S, Buda R, Grigolo B, Vannini F (2001) Autologous chondrocyte transplantation in osteochondral lesions of the ankle joint. Foot Ankle Int 22:513–517

Gibson JN, White MD, Chapman VM, Strachan RK (1992) Arthroscopic lavage and debridement for osteoarthritis of the knee. J Bone Joint Surg Br 74:534–537

Gillespie WJ, O'Connell DL (1992) Arthroscopic lavage of osteoarthritic knees. J Bone Joint Surg Br 74:787–788; author reply 788–789

Goldman RT, Scuderi GR, Kelly MA (1997) Arthroscopic treatment of the degenerative knee in older athletes. Clin Sports Med 16:51–68

Grande DA, Halberstadt C, Naughton G, Schwartz R, Manji R (1997) Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts. J Biomed Mater Res 34:211–220

Grande DA, Mason J, Light E, Dines D (2003) Stem cells as platforms for delivery of genes to enhance cartilage repair. J Bone Joint Surg Am 85-A(Suppl 2):111–116

Grant ME, Ayad S, Kwan APL, Bates GP, Thomas JT, Mcclure J (1988) The structure and synthesis of cartilage collagens. In: Glauert AM (ed) Control of tissue damage. Elsevier Science Publishers, Amsterdam, pp 3–28

Grigolo B, Roseti L, Fiorini M, Fini M, Giavaresi G, Aldini NN, Giardino R, Facchini A (2001) Transplantation of chondrocytes seeded on a hyaluronan derivative (hyaff-11) into cartilage defects in rabbits. Biomaterials 22:2417–2424

Grynpas MD, Alpert B, Katz I, Lieberman I, Pritzker KP (1991) Subchondral bone in osteoarthritis. Calcif Tissue Int 49:20–26

Gu WY, Lai WM, Mow VC (1997) A triphasic analysis of negative osmotic flows through charged hydrated soft tissues. J Biomech 30:71–78

Guevremont M, Martel-Pelletier J, Massicotte F, Tardif G, Pelletier JP, Ranger P, Lajeunesse D, Reboul P (2003) Human adult chondrocytes express hepatocyte growth factor (HGF) isoforms but not HgF: potential implication of osteoblasts on the presence of HGF in cartilage. J Bone Miner Res 18:1073–1081

Guilak F, Mow VC (2000) The mechanical environment of the chondrocyte: a biphasic finite element model of cell-matrix interactions in articular cartilage. J Biomech 33:1663–1673
Guilak F, Jones WR, Ting-Beall HP, Lee GM (1999) The deformation behavior and mechanical properties of chondrocytes in articular cartilage. Osteoarthritis Cartilage 7:59–70
Haisch A, Groger A, Gebert C, Leder K, Ebmeyer J, Sudhoff H, Jovanovic S, Sedlmaier B, Sittinger M (2005) Creating artificial perichondrium by polymer complex membrane macroencapsulation: immune protection and stabilization of subcutaneously transplanted tissue-engineered cartilage. Eur Arch Otorhinolaryngol 262:338–344
Hardingham TE, Fosang AJ (1992) Proteoglycans: many forms and many functions. FASEB J 6:861–870
Hendrickson DA, Nixon AJ, Grande DA, Todhunter RJ, Minor RM, Erb H, Lust G (1994) Chondrocyte-fibrin matrix transplants for resurfacing extensive articular cartilage defects. J Orthop Res 12:485–497
Herzog W, Diet S, Suter E, Mayzus P, Leonard TR, Muller C, Wu JZ, Epstein M (1998) Material and functional properties of articular cartilage and patellofemoral contact mechanics in an experimental model of osteoarthritis. J Biomech 31:1137–1145
Hickey MJ, Ohta I, Shigetomi M, Hurley JV, Kuwata N, O'Brien BM (1994) Vascularized heterotopic osteochondral allografts in a rat model following long-term immunosuppression. J Reconstr Microsurg 10:255–260
Hirsch CA (1944) Contribution to the pathogenesis of chondromalacia of the patella. Acta Chir Scaninavica 83:1–106
Holmdahl DE, Ingelmark BE (1950) The contact between the articular cartilage and the medullary cavities of the bone. Acta Orthop Scand 20:156–165
Homminga GN, Bulstra SK, Bouwmeester PS, van der Linden AJ (1990) Perichondral grafting for cartilage lesions of the knee. J Bone Joint Surg Br 72:1003–1007
Honner R, Thompson RC (1971) The nutritional pathways of articular cartilage. An autoradiographic study in rabbits using 35S injected intravenously. J Bone Joint Surg Am 53:742–748
Huber S, Winterhalter KH, Vaughan L (1988) Isolation and sequence analysis of the glycosaminoglycan attachment site of type IX collagen. J Biol Chem 263:752–756
Huntley JS, Bush PG, McBirnie JM, Simpson AH, Hall AC (2005) Chondrocyte death associated with human femoral osteochondral harvest as performed for mosaicplasty. J Bone Joint Surg Am 87:351–360
Hunziker EB (1999) Biologic repair of articular cartilage. Defect models in experimental animals and matrix requirements. Clin Orthop Relat Res 367:S135–S146
Hurtig M, Pearce S, Warren S, Kalra M, Miniaci A (2001) Arthroscopic mosaic arthroplasty in the equine third carpal bone. Vet Surg 30:228–239
Jensen LK (2008) Hip osteoarthritis: influence of work with heavy lifting, climbing stairs or ladders, or combining kneeling/squatting with heavy lifting. Occup Environ Med 65:6–19
Jones EA, Kinsey SE, English A, Jones RA, Straszynski L, Meredith DM, Markham AF, Jack A, Emery P, McGonagle D (2002) Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum 46:3349–3360
Jurvelin J, Saamanen AM, Arokoski J, Helminen HJ, Kiviranta I, Tammi M (1988) Biomechanical properties of the canine knee articular cartilage as related to matrix proteoglycans and collagen. Eng Med 17:157–162
Kawabe N, Yoshinao M (1991) The repair of full-thickness articular cartilage defects. Immune responses to reparative tissue formed by allogeneic growth plate chondrocyte implants. Clin Orthop Relat Res 268:279–293
Kempson GE, Muir H, Swanson SA, Freeman MA (1970) Correlations between stiffness and the chemical constituents of cartilage on the human femoral head. Biochim Biophys Acta 215:70–77
Key JA (1931) Experimental arthritis: the changes in joints produced by creating defects in the articular cartilage. J Bone Joint Surg Am 13:725–739
Kim HK, Moran ME, Salter RB (1991) The potential for regeneration of articular cartilage in defects created by chondral shaving and subchondral abrasion. An experimental investigation in rabbits. J Bone Joint Surg Am 73:1301–1315

Kim YJ, Sah RL, Grodzinsky AJ, Plaas AH, Sandy JD (1994) Mechanical regulation of cartilage biosynthetic behavior: physical stimuli. Arch Biochem Biophys 311:1–12
Kiviranta P, Lammentausta E, Toyras J, Kiviranta I, Jurvelin JS (2008) Indentation diagnostics of cartilage degeneration. Osteoarthritis Cartilage 16:796–804
Kleemann RU, Schell H, Thompson M, Epari DR, Duda GN, Weiler A (2007) Mechanical behavior of articular cartilage after osteochondral autograft transfer in an ovine model. Am J Sports Med 35:555–563
Knight MM, Lee DA, Bader DL (1998) The influence of elaborated pericellular matrix on the deformation of isolated articular chondrocytes cultured in agarose. Biochim Biophys Acta 1405:67–77
Knudson CB, Knudson W (2001) Cartilage proteoglycans. Semin Cell Dev Biol 12:69–78
Konttinen YT, Mandelin J, Li TF, Salo J, Lassus J, Liljestrom M, Hukkanen M, Takagi M, Virtanen I, Santavirta S (2002) Acidic cysteine endoproteinase cathepsin K in the degeneration of the superficial articular hyaline cartilage in osteoarthritis. Arthritis Rheum 46:953–960
Korhonen RK, Laasanen MS, Toyras J, Rieppo J, Hirvonen J, Helminen HJ, Jurvelin JS (2002) Comparison of the equilibrium response of articular cartilage in unconfined compression, confined compression and indentation. J Biomech 35:903–909
Korkala O, Kuokkanen H (1991) Autogenous osteoperiosteal grafts in the reconstruction of full-thickness joint surface defects. Int Orthop 15:233–237
Kramer J, Bohrnsen F, Schlenke P, Rohwedel J (2006) Stem cell-derived chondrocytes for regenerative medicine. Transplant Proc 38:762–765
Kwan AP, Sear CH, Grant ME (1986) Identification of disulphide-bonded type X procollagen polypeptides in embryonic chick chondrocyte cultures. FEBS Lett 206:267–272
Kwan MK, Coutts RD, Woo SL, Field FP (1989) Morphological and biomechanical evaluations of neocartilage from the repair of full-thickness articular cartilage defects using rib perichondrium autografts: a long-term study. J Biomech 22:921–930
Lai WM, Hou JS, Mow VC (1991) A triphasic theory for the swelling and deformation behaviors of articular cartilage. J Biomech Eng 113:245–258
Lane JM, Weiss C (1975) Review of articular cartilage collagen research. Arthritis Rheum 18:553–562
Laprell H, Petersen W (2001) Autologous osteochondral transplantation using the diamond bone-cutting system (DBCS): 6–12 years' follow-up of 35 patients with osteochondral defects at the knee joint. Arch Orthop Trauma Surg 121:248–253
LeBaron RG, Athanasiou KA (2000) Ex vivo synthesis of articular cartilage. Biomaterials 21:2575–2587
Lee DA, Bader DL (1995) The development and characterization of an in vitro system to study strain-induced cell deformation in isolated chondrocytes. In Vitro Cell Dev Biol 31:828–835
Lee DA, Bader DL (1997) Compressive strains at physiological frequencies influence the metabolism of chondrocytes seeded in agarose. J Orthop Res 15:181–188
Lee DA, Reisler T, Bader DL (2003) Expansion of chondrocytes for tissue engineering in alginate beads enhances chondrocytic phenotype compared to conventional monolayer techniques. Acta Orthop Scand 74:6–15
Li B, Aspden RM (1997) Composition and mechanical properties of cancellous bone from the femoral head of patients with osteoporosis or osteoarthritis. J Bone Miner Res 12:641–651
Li LP, Soulhat J, Buschmann MD, Shirazi-Adl A (1999) Nonlinear analysis of cartilage in unconfined ramp compression using a fibril reinforced poroelastic model. Clin Biomech (Bristol, Avon) 14:673–682
Li WJ, Tuli R, Huang X, Laquerriere P, Tuan RS (2005) Multilineage differentiation of human mesenchymal stem cells in a three-dimensional nanofibrous scaffold. Biomaterials 26:5158–5166
Lievense A, Bierma-Zeinstra S, Verhagen A, Verhaar J, Koes B (2001) Influence of work on the development of osteoarthritis of the hip: a systematic review. J Rheumatol 28:2520–2528
Lin JG, Chen WL (2009) Review: acupuncture analgesia in clinical trials. Am J Chin Med 37:1–18
Linn FC, Sokoloff L (1965) Movement and composition of interstitial fluid of cartilage. Arthritis Rheum 8:481–494

Lipshitz H, Etheredge R 3rd, Glimcher MJ (1976) Changes in the hexosamine content and swelling ratio of articular cartilage as functions of depth from the surface. J Bone Joint Surg Am 58:1149–1153

Livesley PJ, Doherty M, Needoff M, Moulton A (1991) Arthroscopic lavage of osteoarthritic knees. J Bone Joint Surg Br 73:922–926

Lusse S, Claassen H, Gehrke T, Hassenpflug J, Schunke M, Heller M, Gluer CC (2000) Evaluation of water content by spatially resolved transverse relaxation times of human articular cartilage. Magn Reson Imaging 18:423–430

Ma HL, Chen TH, Low-Tone Ho L, Hung SC (2005) Neocartilage from human mesenchymal stem cells in alginate: implied timing of transplantation. J Biomed Mater Res A 74:439–446

Mahomed MN, Beaver RJ, Gross AE (1992) The long-term success of fresh, small fragment osteochondral allografts used for intraarticular post-traumatic defects in the knee joint. Orthopedics 15:1191–1199

Mak AF (1986) The apparent viscoelastic behavior of articular cartilage–the contributions from the intrinsic matrix viscoelasticity and interstitial fluid flows. J Biomech Eng 108:123–130

Malinin T, Ouellette EA (2000) Articular cartilage nutrition is mediated by subchondral bone: a long-term autograft study in baboons. Osteoarthritis Cartilage 8:483–491

Mankin HJ (1962) Localization of tritiated thymidine in articular cartilage of rabbits: II. Repair in immature cartilage. J Bone Joint Surg Am 44:688–698

Mankin HJ (1974a) The reaction of articular cartilage to injury and osteoarthritis (first of two parts). N Engl J Med 291:1285–1292

Mankin HJ (1974b) The reaction of articular cartilage to injury and osteoarthritis (second of two parts). N Engl J Med 291:1335–1340

Mankin HJ (1982) The response of articular cartilage to mechanical injury. J Bone Joint Surg Am 64:460–466

Maroudas A (1976) Transport of solutes through cartilage: permeability to large molecules. J Anat 122:335–347

Maroudas A (1979) Physicochemical properties of articular cartilage. In: Freeman MAR (ed) Adult articular cartilage. Kent/Pitman Medical Publishing Co. Ltd., Tunbridge Wells, pp 215–290

Martin I, Obradovic B, Freed LE, Vunjak-Novakovic G (1999) Method for quantitative analysis of glycosaminoglycan distribution in cultured natural and engineered cartilage. Ann Biomed Eng 27:656–662

Masuoka K, Asazuma T, Ishihara M, Sato M, Hattori H, Ishihara M, Yoshihara Y, Matsui T, Takase B, Kikuchi M et al (2005) Tissue engineering of articular cartilage using an allograft of cultured chondrocytes in a membrane-sealed atelocollagen honeycomb-shaped scaffold (ACHMS scaffold). J Biomed Mater Res B Appl Biomater 75:177–184

Matsuno H, Nakamura H, Katayama K, Hayashi S, Kano S, Yudoh K, Kiso Y (2009) Effects of an oral administration of glucosamine-chondroitin-quercetin glucoside on the synovial fluid properties in patients with osteoarthritis and rheumatoid arthritis. Biosci Biotechnol Biochem 73:288–292

McCutchen CW (1962) The frictional properties of animal joints. Wear 5:1–17

McKibbin B, Holdsworth FW (1966) The nutrition of immature joint cartilage in the lamb. J Bone Joint Surg Br 48:793–803

Meachim G, Stockwell RA (1979) The matrix. In: Freeman MAR (ed) Adult articular cartilage. Kent/Pitman Medical Publishing Co. Ltd., Tunbridge Wells, pp 600–610

Meisenberg G, Simmons WH (1998) Principles of medical biochemistry. Mosby Inc., St. Louis

Menche DS, Frenkel SR, Blair B, Watnik NF, Toolan BC, Yaghoubian RS, Pitman MI (1996) A comparison of abrasion burr arthroplasty and subchondral drilling in the treatment of full-thickness cartilage lesions in the rabbit. Arthroscopy 12:280–286

Messner K, Fahlgren A, Ross I, Andersson B (2000) Simultaneous changes in bone mineral density and articular cartilage in a rabbit meniscectomy model of knee osteoarthrosis. Osteoarthritis Cartilage 8:197–206

Meyers MH, Akeson W, Convery FR (1989) Resurfacing of the knee with fresh osteochondral allograft. J Bone Joint Surg Am 71:704–713

Micheli LJ, Browne JE, Erggelet C, Fu F, Mandelbaum B, Moseley JB, Zurakowski D (2001) Autologous chondrocyte implantation of the knee: multicenter experience and minimum 3-year follow-up. Clin J Sport Med 11:223–228

Minas T (1998) Chondrocyte implantation in the repair of chondral lesions of the knee: economics and quality of life. Am J Orthop (Belle Mead) 27:739–744

Minguell JJ, Erices A, Conget P (2001) Mesenchymal stem cells. Exp Biol Med (Maywood) 226:507–520

Mitchell N, Shepard N (1987) Effect of patellar shaving in the rabbit. J Orthop Res 5:388–392

Moldovan F, Pelletier JP, Hambor J, Cloutier JM, Martel-Pelletier J (1997) Collagenase-3 (matrix metalloprotease 13) is preferentially localized in the deep layer of human arthritic cartilage in situ: in vitro mimicking effect by transforming growth factor beta. Arthritis Rheum 40:1653–1661

Mollenhauer J, Bee JA, Lizarbe MA, von der Mark K (1984) Role of anchorin CII, a 31,000-mol-wt membrane protein, in the interaction of chondrocytes with type II collagen. J Cell Biol 98:1572–1579

Mooney DJ, Baldwin DF, Suh NP, Vacanti JP, Langer R (1996) Novel approach to fabricate porous sponges of poly (D, L-lactic-co-glycolic acid) without the use of organic solvents. Biomaterials 17:1417–1422

Moran ME, Kim HK, Salter RB (1992) Biological resurfacing of full-thickness defects in patellar articular cartilage of the rabbit. Investigation of autogenous periosteal grafts subjected to continuous passive motion. J Bone Joint Surg Br 74:659–667

Moseley JB Jr, Wray NP, Kuykendall D, Willis K, Landon G (1996) Arthroscopic treatment of osteoarthritis of the knee: a prospective, randomized, placebo-controlled trial. Results of a pilot study. Am J Sports Med 24:28–34

Mow VC, Kuei SC, Lai WM, Armstrong CG (1980) Biphasic creep and stress relaxation of articular cartilage in compression? Theory and experiments. J Biomech Eng 102:73–84

Mow VC, Holmes MH, Lai WM (1984) Fluid transport and mechanical properties of articular cartilage: a review. J Biomech 17:377–394

Mow VC, Ratcliffe A, Poole AR (1992) Cartilage and diarthrodial joints as paradigms for hierarchical materials and structures. Biomaterials 13:67–97

Muir H (1980) The chemistry of the ground substance of joint cartilage. In: Sokolff L (ed) The joints and synovial fluid, vol 2. Academic, New York, pp 27–94

Muir H (1995) The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. Bioessays 17:1039–1048

Muir H, Bullough P, Maroudas A (1970) The distribution of collagen in human articular cartilage with some of its physiological implications. J Bone Joint Surg Br 52:554–563

Nakase T, Kaneko M, Tomita T, Myoui A, Ariga K, Sugamoto K, Uchiyama Y, Ochi T, Yoshikawa H (2000) Immunohistochemical detection of cathepsin D, K, and L in the process of endochondral ossification in the human. Histochem Cell Biol 114:21–27

Nehrer S, Spector M, Minas T (1999) Histologic analysis of tissue after failed cartilage repair procedures. Clin Orthop Relat Res 365:149–162

Niederauer GG, Slivka MA, Leatherbury NC, Korvick DL, Harroff HH, Ehler WC, Dunn CJ, Kieswetter K (2000) Evaluation of multiphase implants for repair of focal osteochondral defects in goats. Biomaterials 21:2561–2574

Nishida K, Inoue H, Murakami T (1995) Immunohistochemical demonstration of fibronectin in the most superficial layer of normal rabbit articular cartilage. Ann Rheum Dis 54:995–998

Nishitani K, Nakagawa Y, Gotoh T, Kobayashi M, Nakamura T (2008) Intraoperative acoustic evaluation of living human cartilage of the elbow and knee during mosaicplasty for osteochondritis dissecans of the elbow: an in vivo study. Am J Sports Med 36:2345–2353

O'Byrne E, Pellas T, Laurent D (2003) Qualitative and quantitative in vivo assessment of articular cartilage using magnetic resonance imaging. Novartis Found Symp 249:190–198; discussion 198–202, 234–198, 239–141

Ohlsen L (1976) Cartilage formation from free perichondrial grafts: an experimental study in rabbits. Br J Plast Surg 29:262–267

Ossendorf C, Kaps C, Kreuz PC, Burmester GR, Sittinger M, Erggelet C (2007) Treatment of posttraumatic and focal osteoarthritic cartilage defects of the knee with autologous polymer-based three-dimensional chondrocyte grafts: 2-year clinical results. Arthritis Res Ther 9:R41

Ostrander RV, Goomer RS, Tontz WL, Khatod M, Harwood FL, Maris TM, Amiel D (2001) Donor cell fate in tissue engineering for articular cartilage repair. Clin Orthop Relat Res 389:228–237

Owens S, Wagner P, Vangsness CT Jr (2004) Recent advances in glucosamine and chondroitin supplementation. J Knee Surg 17:185–193

Pavesio A, Abatangelo G, Borrione A, Brocchetta D, Hollander AP, Kon E, Torasso F, Zanasi S, Marcacci M (2003) Hyaluronan-based scaffolds (Hyalograft C) in the treatment of knee cartilage defects: preliminary clinical findings. Novartis Found Symp 249:203–217; discussion 229–233, 234–208, 239–241

Pedersen MS, Moghaddam AZ, Bak K, Koch JS (1995) The effect of bone drilling on pain in gonarthrosis. Int Orthop 19:12–15

Perka C, Schultz O, Spitzer RS, Lindenhayn K, Burmester GR, Sittinger M (2000) Segmental bone repair by tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. Biomaterials 21:1145–1153

Peterson L, Minas T, Brittberg M, Lindahl A (2003) Treatment of osteochondritis dissecans of the knee with autologous chondrocyte transplantation: results at two to ten years. J Bone Joint Surg Am 85-A(Suppl 2):17–24

Pfander D, Cramer T, Weseloh G, Pullig O, Schuppan D, Bauer M, Swoboda B (1999) Hepatocyte growth factor in human osteoarthritic cartilage. Osteoarthritis Cartilage 7:548–559

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science (New York) 284:143–147

Poole CA (1997) Articular cartilage chondrons: form, function and failure. J Anat 191(Pt 1):1–13

Poole AR, Pidoux I, Reiner A, Rosenberg L (1982) An immunoelectron microscope study of the organization of proteoglycan monomer, link protein, and collagen in the matrix of articular cartilage. J Cell Biol 93:921–937

Pugh JW, Radin EL, Rose RM (1974) Quantitative studies of human subchondral cancellous bone. Its relationship to the state of its overlying cartilage. J Bone Joint Surg Am 56:313–321

Pulkkinen L, Kainulainen K, Krusius T, Makinen P, Schollin J, Gustavsson KH, Peltonen L (1990) Deficient expression of the gene coding for decorin in a lethal form of Marfan syndrome. J Biol Chem 265:17780–17785

Quinn TM, Morel V, Meister JJ (2001) Static compression of articular cartilage can reduce solute diffusivity and partitioning: implications for the chondrocyte biological response. J Biomech 34:1463–1469

Radin EL, Rose RM (1986) Role of subchondral bone in the initiation and progression of cartilage damage. Clin Orthop Relat Res 213:34–40

Radin EL, Paul IL, Lowy M (1970) A comparison of the dynamic force transmitting properties of subchondral bone and articular cartilage. J Bone Joint Surg Am 52:444–456

Ratcliffe A, Mow VC (1976) Structure and function of articular cartilage. In: Comper WD (ed) Extracellular matrix. Harwood Academic Publisher, Amsterdam, pp 234–302

Reboul P, Pelletier JP, Tardif G, Benderdour M, Ranger P, Bottaro DP, Martel-Pelletier J (2001) Hepatocyte growth factor induction of collagenase 3 production in human osteoarthritic cartilage: involvement of the stress-activated protein kinase/c-Jun N-terminal kinase pathway and a sensitive p38 mitogen-activated protein kinase inhibitor cascade. Arthritis Rheum 44:73–84

Redman SN, Dowthwaite GP, Thomson BM, Archer CW (2004) The cellular responses of articular cartilage to sharp and blunt trauma. Osteoarthritis Cartilage 12:106–116

Reinhold T, Witt CM, Jena S, Brinkhaus B, Willich SN (2008) Quality of life and cost-effectiveness of acupuncture treatment in patients with osteoarthritis pain. Eur J Health Econ 9:209–219

Repo RU, Finlay JB (1977) Survival of articular cartilage after controlled impact. J Bone Joint Surg Am 59:1068–1076

Ringe J, Sittinger M (2009) Tissue engineering in the rheumatic diseases. Arthritis Res Ther 11:211
Risbud MV, Sittinger M (2002) Tissue engineering: advances in in vitro cartilage generation. Trends Biotechnol 20:351–356
Roddy E, Zhang W, Doherty M (2005) Aerobic walking or strengthening exercise for osteoarthritis of the knee? A systematic review. Ann Rheum Dis 64:544–548
Romeo AA, Cole BJ, Mazzocca AD, Fox JA, Freeman KB, Joy E (2002) Autologous chondrocyte repair of an articular defect in the humeral head. Arthroscopy 18:925–929
Rosenberg L (1971) Chemical basis for the histological use of safranin O in the study of articular cartilage. J Bone Joint Surg Am 53:69–82
Ruoslahti E, Yamaguchi Y (1991) Proteoglycans as modulators of growth factor activities. Cell 64:867–869
Russlies M, Behrens P, Wunsch L, Gille J, Ehlers EM (2002) A cell-seeded biocomposite for cartilage repair. Ann Anat 184:317–323
Ryan MC, Sandell LJ (1990) Differential expression of a cysteine-rich domain in the amino-terminal propeptide of type II (cartilage) procollagen by alternative splicing of mRNA. J Biol Chem 265:10334–10339
Salter DM (1993) Tenascin is increased in cartilage and synovium from arthritic knees. Br J Rheumatol 32:780–786
Sams AE, Minor RR, Wootton JA, Mohammed H, Nixon AJ (1995) Local and remote matrix responses to chondrocyte-laden collagen scaffold implantation in extensive articular cartilage defects. Osteoarthritis Cartilage 3:61–70
Samuel RE, Lee CR, Ghivizzani SC, Evans CH, Yannas IV, Olsen BR, Spector M (2002) Delivery of plasmid DNA to articular chondrocytes via novel collagen-glycosaminoglycan matrices. Hum Gene Ther 13:791–802
Schell H, Lienau J, Kleemann RU, Schlichting K, Taylor WR, Weiler A, Duda GN (2007) Crushed bone grafts and a collagen membrane are not suitable for enhancing cartilage quality in the regeneration of osteochondral defects–an in vivo study in sheep. J Biomech 40(Suppl 1):S64–S72
Schlichting K, Schell H, Kleemann RU, Schill A, Weiler A, Duda GN, Epari DR (2008) Influence of scaffold stiffness on subchondral bone and subsequent cartilage regeneration in an ovine model of osteochondral defect healing. Am J Sports Med 36:2379–2391
Seradge H, Kutz JA, Kleinert HE, Lister GD, Wolff TW, Atasoy E (1984) Perichondrial resurfacing arthroplasty in the hand. J Hand Surg 9:880–886
Sirlin CB, Brossmann J, Boutin RD, Pathria MN, Convery FR, Bugbee W, Deutsch R, Lebeck LK, Resnick D (2001) Shell osteochondral allografts of the knee: comparison of mr imaging findings and immunologic responses. Radiology 219:35–43
Sittinger M, Bujia J, Minuth WW, Hammer C, Burmester GR (1994) Engineering of cartilage tissue using bioresorbable polymer carriers in perfusion culture. Biomaterials 15:451–456
Sittinger M, Hutmacher DW, Risbud MV (2004) Current strategies for cell delivery in cartilage and bone regeneration. Curr Opin Biotechnol 15:411–418
Sledge SL (2001) Microfracture techniques in the treatment of osteochondral injuries. Clin Sports Med 20:365–377
Solchaga LA, Gao J, Dennis JE, Awadallah A, Lundberg M, Caplan AI, Goldberg VM (2002) Treatment of osteochondral defects with autologous bone marrow in a hyaluronan-based delivery vehicle. Tissue Eng 8:333–347
Soren A (1965) Treatment of musculoskeletal disorders with ultrasound. J Occup Med 7:434–438
Stevenson S (1987) The immune response to osteochondral allografts in dogs. J Bone Joint Surg Am 69:573–582
Stevenson S, Dannucci GA, Sharkey NA, Pool RR (1989) The fate of articular cartilage after transplantation of fresh and cryopreserved tissue-antigen-matched and mismatched osteochondral allografts in dogs. J Bone Joint Surg Am 71:1297–1307
Stockwell RA, Meachim G (1979) The chondrocyte. In: Freeman MAR (ed) Adult articular cartilage. Kent/Pitman Medical Publishing Co. Ltd., Tunbridge Wells, pp 600–610

Tamai N, Myoui A, Hirao M, Kaito T, Ochi T, Tanaka J, Takaoka K, Yoshikawa H (2005) A new biotechnology for articular cartilage repair: subchondral implantation of a composite of interconnected porous hydroxyapatite, synthetic polymer (PLA-PEG), and bone morphogenetic protein-2 (rhBMP-2). Osteoarthritis Cartilage 13:405–417

Temenoff JS, Mikos AG (2000) Review: tissue engineering for regeneration of articular cartilage. Biomaterials 21:431–440

Tew SR, Kwan AP, Hann A, Thomson BM, Archer CW (2000) The reactions of articular cartilage to experimental wounding: role of apoptosis. Arthritis Rheum 43:215–225

Thompson RC Jr (1975) An experimental study of surface injury to articular cartilage and enzyme responses within the joint. Clin Orthop Relat Res 107:239–248

Tibesku CO, Szuwart T, Kleffner TO, Schlegel PM, Jahn UR, Van Aken H, Fuchs S (2004) Hyaline cartilage degenerates after autologous osteochondral transplantation. J Orthop Res 22:1210–1214

Torzilli PA, Mow VC (1976) On the fundamental fluid transport mechanisms through normal and pathological articular cartilage during function–I the formulation. J Biomech 9:541–552

Towheed TE, Maxwell L, Judd MG, Catton M, Hochberg MC, Wells G (2006) Acetaminophen for osteoarthritis. Cochrane Database Syst Rev (Online) CD004257

Vangsness CT, Smith CF (1995) Arthroscopic shoulder surgery with three different laser systems: an evaluation of laser applications. Arthroscopy 11:696–700

Vogel KG, Paulsson M, Heinegard D (1984) Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. Biochem J 223:587–597

von der Mark K, Gauss V, von der Mark H, Muller P (1977) Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature 267:531–532

Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI, Goldberg VM (1994) Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Joint Surg Am 76:579–592

Wakitani S, Imoto K, Yamamoto T, Saito M, Murata N, Yoneda M (2002) Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthritis Cartilage 10:199–206

Wegman A, van der Windt D, van Tulder M, Stalman W, de Vries T (2004) Nonsteroidal antiinflammatory drugs or acetaminophen for osteoarthritis of the hip or knee? A systematic review of evidence and guidelines. J Rheumatol 31:344–354

Weightman B, Kempson GE (1979) Load cartilage. In: Freeman MAR (ed) Adult articular cartilage. Pitman Medical Publishing Co. Ltd., Tunbridge Wells, pp 291–331

Weisser J, Rahfoth B, Timmermann A, Aigner T, Brauer R, von der Mark K (2001) Role of growth factors in rabbit articular cartilage repair by chondrocytes in agarose. Osteoarthritis Cartilage 9(Suppl A):S48–S54

Welch V, Brosseau L, Peterson J, Shea B, Tugwell P, Wells G (2001) Therapeutic ultrasound for osteoarthritis of the knee. Cochrane Database Syst Rev (Online) CD003132

Whiteside RA, Jakob RP, Wyss UP, Mainil-Varlet P (2005) Impact loading of articular cartilage during transplantation of osteochondral autograft. J Bone Joint Surg Br 87:1285–1291

Wong M, Ponticiello M, Kovanen V, Jurvelin JS (2000) Volumetric changes of articular cartilage during stress relaxation in unconfined compression. J Biomech 33:1049–1054

Wyre RM, Downes S (2000) An in vitro investigation of the PEMA/THFMA polymer system as a biomaterial for cartilage repair. Biomaterials 21:335–343

Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM, Johnstone B (1998) The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. J Bone Joint Surg Am 80:1745–1757

Zhang W, Jones A, Doherty M (2004) Does paracetamol (acetaminophen) reduce the pain of osteoarthritis? A meta-analysis of randomised controlled trials. Ann Rheum Dis 63:901–907

Chapter 42
Muscle and Ligament Regeneration

Thomas Mittlmeier and Ioannis Stratos

Abstract Muscle injury and degenerative muscle diseases are disabling conditions that are currently challenging orthopedic surgeons, neurologists and specialists in rehabilitative medicine. Upon traumatic or degenerative changes in the structure of the muscle, regeneration befalls mainly by increased proliferation of satellite cells. If the injury is extensive fibrosis and scar tissue formation occurs. Till now various alternative therapeutic ways have been proposed to boost muscle regeneration. These methods include the use of growth factors, antioxidative therapeutic approaches, cell based therapy and cell transplantation as well as the use of scaffolds. Growth factors, antioxidative substances and endogenous polypeptides can not only influence but also control the natural repair processes by acting on different intracellular pathways. Cell orientated therapies have been popular in muscle regeneration mainly because small quantities of cells are needed to achieve therapeutic effects. Transplantation of stem cells, myoblasts or genetically modified cells, have been used after injury to restore muscle structure and function. Furthermore scaffolds have been used to repair muscle defects and to generate new muscle fibers.

Similar approaches have been made for regeneration of ligaments. There are a number of cell sources that are potentially helpful for cell mediated tissue regeneration. Scaffolds provide temporary mechanical support and can carry cells that promote the ligament regeneration. Furthermore growth factors can be used to stimulate ligament healing and accelerate regeneration mainly by modulating the proliferation.

T. Mittlmeier, M.D. (✉) • I. Stratos, M.D.
Department of Trauma and Reconstructive Surgery, Rostock University, Medical Center, Schillingallee 35, 18057 Rostock, Germany
e-mail: thomas.mittlmeier@med.uni-rostock.de

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_42,

42.1 Muscle Regeneration

42.1.1 The Skeletal Muscle: Injury and Repair

Muscle is defined as the anatomical construct of animals with the ability to contract. The skeletal muscle tissue derives from paraxial mesoderm. During development, myoblasts migrate to their destinations, where they fuse to form elongated skeletal muscle cells. Muscle cells contain contractile filaments that move past each other and change the size of the cell. The human body consists of more than 600 different muscles, which allow us to breath, to go and to perform daily tasks. About 40% of the total body mass in males are muscles whereas 25% in females, only.

Over a short period of time after muscle injury ischemic events as well as inflammatory damage of myocytes and interstitial muscle tissue cells occurs. Immediately after, the nutritive microvascular perfusion of the muscle gradually surceases and the inflammatory response with leukocyte endothelial cell interaction enlarges. Subsequently, normal function of the endothelial cell barrier is disrupted and interstitial muscle edema establishes (Oestern and Tscherne 1983). At later time points after muscle injury tissue regeneration is characterized by three phases (Järvinen et al. 2005): (1) Destruction phase: rupture and necrosis of muscle cells, hematoma formation and leukocyte cell infiltration; (2) Regeneration phase: phagocytosis of the necrotic tissue, regeneration of myofibers and formation of fibrotic tissue, capillary incorporation as well as angiogenesis into the traumatic tissue; (3) Remodeling phase: reconstruction and regeneration of myofibers, increase of the breaking strength of the traumatized tissue, reorganization of the scar tissue and functional remodeling of the muscle.

Similar pathological changes occur not only after muscle injury but also in dystrophic muscle disease. Muscular dystrophies are a heterogeneous group of hereditary diseases affecting both children and adults, and are characterized by muscle wasting and weakness. Degenerative muscle diseases, like muscular dystrophies, involve cycles of segmental necrosis and regeneration. The muscle tissue is thereby characterized by fiber size variability, necrosis, regeneration, inflammation and connective tissues deposition (Ciciliot and Schiaffino 2009).

42.1.2 Regenerative Capacity of Skeletal Muscle

The skeletal muscle is an irreversibly post-mitotic tissue that has under normal conditions a very low mitotic activity. Under certain conditions and in response to various stimuli like very mild trauma, regenerative cascades in the muscle become activated in order to restore the injured tissue. Under normal conditions muscle regeneration is initiated subsequently to muscle injury.

The most important cells during muscular regeneration are satellite cells. Satellite cells are undifferentiated reserve cells, which are located in the gap between

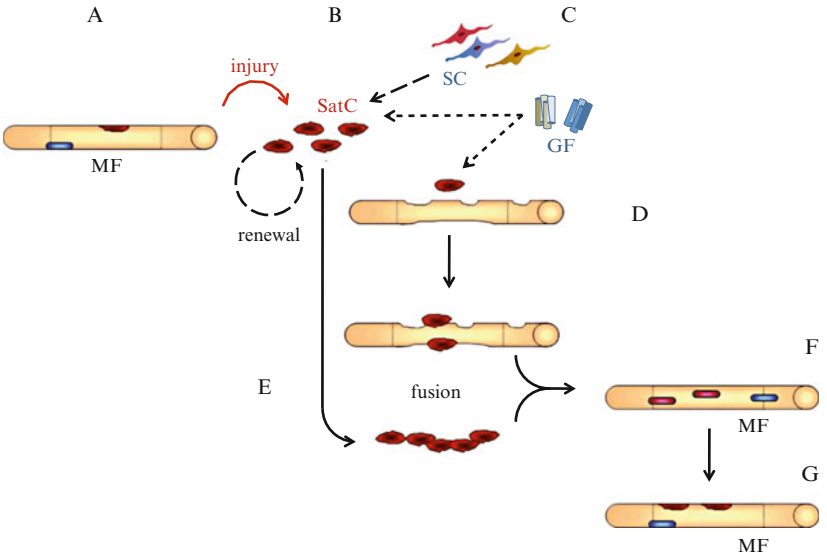


Fig. 42.1 The muscle regeneration: (*A*) If a normal myofiber (*MF*) is injured (*B*) satellite cells (*SatC*) become activated and start to proliferate. Circulating stem cells as well as local stem cells from the skeletal muscle (*SC*) differentiate to satellite cells (*C*) and participate during the repair process in the muscle regeneration. The satellite cells fuse with the injured myofibers (*D*) or together (*E*) and form new myofibers with central nuclei (*F*). Growth factors (*GF*) control the procedure and enhance the regeneration. At a later time point the newly formed nuclei move away from the center of the cell and reside beneath the cell membrane (*G*)

basal lamina and plasma membrane of each individual myofiber (Mauro 1961). In response to muscle injuries satellite cells proliferate, differentiate into myoblasts and finally fuse together to form a multinucleated myotube. The newly formed myotubes fuse with the injured myofiber that has survived the initial trauma.

In the mature muscle tissue two major populations of satellite cells reside. The first population of satellite cells directly repairs the injured tissue by proliferation and differentiation into myoblasts immediately after muscle injury. The second population of satellite cells proliferates after muscle injury and replenishes the existing satellite cell pool by undergoing cell divisions before differentiation. Furthermore, it is known that post trauma, stem cells from the bone marrow but also muscle residual stem cells are migrating to the injured skeletal muscle and refill the satellite cell pool. It is assumed that some satellite cells are capable to differentiate not only into myogenic cell lineages but almost any cells lineage of mesenchymal origin (Fig. 42.1).

However, this regenerative capacity is not infinite, as fatigue of the satellite cell population is an important factor during the regenerative process especially in patients with congenital myopathies such as Duchenne muscular dystrophy. Although the endogenous regenerative capacity of skeletal muscle can conventionally be supported by physical means (like rest, ice, cooling and elevation of

the injured limb) the recovery is not always ample. Novel therapeutic strategies can be applied to restore, improve and maintain the function of the muscle tissue healing during injury, disease, age and congenital defects.

42.1.3 Growth Factors and Muscle Boosters

Muscular regeneration is a crucial biological process, which occurs during the natural repair cycle. The recognition of biologically active proteins, which can enhance the repair process, is still under investigation. The ideal muscle booster would be a molecule that does not have side effects and can specifically and successfully be delivered into the injured muscle. Further goals of the generative therapies include the maintenance of pre-traumatic muscle mass, reduction of the post-traumatic muscle loss and up-regulation of muscle regeneration. Up to now, many factors have been recognized to control natural repair processes by acting on different intracellular pathways.

IGF-I and IGF-II (Insulin like Growth Factor I and II): The IGF-l and IGF-II increases the satellite cell proliferation, restores muscle mass after injury, mobilizes non-muscle stem cells and improves regeneration in aged myopathy related skeletal muscle (Husmann et al. 1996; Singleton and Feldman 2001; Barton et al. 2002; McKay et al. 2008). Furthermore IGF-I seems to utilize pathways in regulating the satellite cell pool.

TGF (Transforming growth factor): The TGF plays a crucial role in regulating the repair and remodeling following tissue injury. Further TGF mediates many biological actions on extracellular matrix components (Husmann et al. 1996).

FGF (Fibroblast growth factor): The fibroblast growth factor family participates in the muscle regeneration and has been suggested as a potent activator of myocytes satellite cells (Charge and Rudnicki 2004). Especially FGF-2 and FGF-6 accelerate muscle regeneration (Israeli et al. 2004; Li et al. 2010).

HGF (Hepatocyte growthfactor): The HGF plays a key role during the early stage of muscle regeneration. It promotes the satellite cell proliferation and migration into the site of injury as well as the stimulation of quiescence satellite cells (Charge and Rudnicki 2004).

MSTN (Myostatin): Myostatin is a potent negative regulator of skeletal muscle growth and member of the tumor growth factor-beta family. Disruption of the myostatin gene causes a combination of hypertrophy and hyperplasia which induces a remarkable increase in muscle mass. The depletion or inactivation of myostatin leads to a significant improvement in muscle regeneration processes, especially in degenerative diseases, mainly through stimulation of satellite cell proliferation and differentiation (Wagner 2005).

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells): The NF-κB is a protein complex that controls the transcription of DNA. NF-κB

promotes proliferation, inhibits differentiation and modulates muscle development (Peterson and Guttridge 2008). Further, it modulates immune response, inflammation and cell survival in skeletal muscle disease (Mourkioti and Rosenthal 2008).

EPO (Erythropoietin), G-CSF (Granulocyte-Colony Stimulating Factor) and PDGF (Platelet Derived Growth Factor): Are endogenic polypeptides with pleotropic actions on a variety of hematopoietic and non-hematopoietic cells. In relation to muscle regeneration EPO has been recognized as anti-apoptotic and tissue protective protein which increases the proliferation of local cells and improves muscle function (Rotter et al. 2008) after muscle injury as well as after muscle ischemia (Kim and Hong 2007). G-CSF has been found to increase satellite cell proliferation and reduce cell apoptosis after muscle injury resulting in faster and better muscle restoration (Stratos et al. 2007; Naito et al. 2009). The PDGF seems to increase myoblast proliferation, acts chemotactic for satellite cells and stimulates the angiogenesis (Husmann et al. 1996).

LIF (Leukemia Inhibitory Factor): The LIF is an interleukin 6 class cytokine that affects satellite cell growth and development as well as proliferation and differentiation after injury (Charge and Rudnicki 2004; Karalaki et al. 2009).

ß2 adrenergic agonists: ß2 adrenergic agonists stimulate the muscle growth and impede the muscle loss. They further suppress protein degradation (Yimlamai et al. 2005), provoke neuronal growth and activity as well as enhance the replacement of muscle specific proteins (Arai et al. 2006).

Calcineurin: Calcineurin is a calcium and calmodulin-dependent serine/threonine protein phosphatase, which mediates myotube differentiation, enhances myoblast recruitment and ameliorates injury to the dystrophic muscle (Mitchell and Pavlath 2002).

Melatonin: Melatonin is an indolamine that enhances muscle force, reduces apoptosis and impedes inflammation after muscle injury (Stratos et al. 2012b). Additionally it has been shown that Melatonin protects against ischemia/reperfusion injury in skeletal muscle (Erkanli et al. 2005) by acting as a potent antioxidative substance (Halici et al. 2004).

Furthermore, various other substances have been described to promote muscle regeneration like caplains (calcium dependent proteases) as well as diverse steroids and hormones that seem to promote myoblast recruitment, enhance proliferation and induce muscle growth.

42.1.4 Antioxidative and Antiapoptotic Therapy

Antioxidative substances have been used with intent to prevent oxidative stress and the experimental results showed mixed success. Antioxidants may potentially reduce certain types of muscle damage but supplementation probably cannot totally prevent muscle injury and degeneration. Ubiquinone seems to stabilize the cell

membrane after injury (Kon et al. 2007) and to stop the ischemia reperfusion injury (Bolcal et al. 2007). Vitamin C is a major antioxidant and has important functions in connective tissue and immune function. Vitamin C application before muscle injury preserves muscle function and reduces the local infiltration and edema (Kearns et al. 2004). Furthermore, Vitamin C prevents microvascular dysfunction in the skeletal muscle of septic rats (Armour et al. 2001) and decreases the exercise-induced increase in the rate of lipid peroxidation (Evans 2000). Alpha Lipoic acid (an essential cofactor for many enzyme complexes) and Isoflavonoids (a group of plant chemicals) have beneficial effects upon oxidative-induced damage of muscle tissue. Supplementation with vitamin E increases muscle force after injury (Warren et al. 1992) reduces the levels of protein oxidation in the skeletal muscle and lessens the exercise-induced lipid damage (Reznick et al. 1992). Several groups have tested combined supplementation treatments in relation to exercise-induced muscle damage. Combinations have included mainly vitamins E and C as well as other antioxidants (Baskin et al. 2000; You et al. 2005).

Antiapoptotic strategies have also shown enhanced muscle regeneration after injury. Additionally, it is postulated that inhibition of caspase activation could be a potential therapeutic target during katabolic events, trauma and disease (Jackman and Kandarian 2004). Inhibition of caspase-mediated apoptosis supported the functional restoration of the injured muscle by amplifying muscle force and furthermore promoted the survival of the injured myofibers by decreased muscle atrophy (Stratos et al. 2012a). Interestingly, Hu et al. reported that transgenic mice, which selectively express the endogenous caspase inhibitor X-chromosome-linked inhibitor of apoptosis protein (XIAP) in skeletal muscles, have hypertrophic peripheral muscles compared to wild-type animals (Hu et al. 2010).

42.1.5 Cell Therapy and Muscle Regeneration

Till now, cell orientated therapies have been introduced which endorse muscular regeneration. Stem cell therapy is an attractive method to treat both muscle injury and dystrophic muscle tissue, because a small quantity of cells is required to achieve therapeutic effects (Price et al. 2007). A current classification of non muscle stem cells involved in muscle regeneration includes (1) non muscle stem cells from the ectoderm and endoderm e.g. neural stem cells (Galli et al. 2000) (2) non muscle stem cells from the hematopoietic system and (3) non muscle stem cells from the solid mesoderm.

Non muscle stem cells from the hematopoietic system: Possible ways to induce muscular regeneration by non muscle stem cells from the hematopoietic system includes the usage of cells from the bone marrow (Ferrari et al. 1998) or transplantation of bone marrow side population cells (Motohashi et al. 2008). Furthermore, non muscle stem cells from the hematopoietic system expressing AC133+ are able to undergo myogenesis when cocultured with myogenic cells or when transplanted in-vivo (Torrente et al. 2004). Using genetically marked bone marrow derived cells in a mouse model Ferrari and colleagues could show that this cell population could

migrate into areas of induced muscle degeneration. Furthermore, these bone marrow derived cells could participate in the regeneration of damaged muscle fibers by undergoing myogenic differentiation (Ferrari et al. 1998). Differentiated myotubes can be formed in-vitro as well as in-vivo (Jiang et al. 2002; Muguruma et al. 2003) by using multipotent adult progenitor progenitor cells. Furthermore, it is known that in response to injury bone marrow-derived cells are not only capable to differentiate into satellite cells but also to fuse with the existing damaged myofibers and to regenerate the muscle fibers (LaBarge and Blau 2002; Corbel et al. 2003). For further support of his statements a parabiotic animal model was used. During these experiments, the vascular systems of observed mice were surgically joined. The authors could demonstrate that bone marrow-derived cells formed skeletal myofibers in injured and physiologically stressed muscle (Sherwood et al. 2004).

Non muscle stem cells from the solid mesoderm: Non muscle stem cells from solid mesoderm which participate to the muscular regeneration include mesenchymal stem cells (MSC). Isolated MSC from the bone marrow can be expanded in a cell culture, and differentiate into a variety of cells of mesenchymal origin including skeletal muscle. MSC have been used to restore the function and anatomy of degenerated peripheral skeletal muscle in cases of myopathy and other congenital muscle diseases (Dezawa et al. 2005). The injection of MSC into the muscle of mdx mice, led to the formation of functional myofibers and satellite cells (De Bari et al. 2003). Furthermore, it has been described that MSC are being mobilized into the peripheral blood in response to injury and to further migrate from the blood across endothelial cells into the injured tissue. Transplantation of MSC into the muscle after injury causes a site-specific differentiation of the MSC into myocytes. Multipotent adult progenitor cells can be differentiated in-vitro in mesenchymallineages and participate in the muscular regeneration. Muscle derived stem cells reside in adult skeletal muscle, express CD34 as well as Sca-l and improve skeletal regeneration (Qu et al. 1998). Mesoangioblasts have a myogenic potential in culture and are capable of ameliorating the symptoms of a number of differing skeletal muscle pathologies (Otto et al. 2009). Further cells with myogenic capacity include endothelial progenitor cells (Takahashi et al. 1999), stem cells from adipose tissue (Rodriguez et al. 2005) as well as stem cells from synovium (De Bari et al. 2003).

Myoblasts: Up to now, many therapeutic attempts have been proposed to cure degenerative muscle disease using myoblasts. Based upon early experimental findings on mdx mice, dystrophin expression was restored after intramuscular transplantation of myoblasts. Irintchev et al. (1997) applied in 1997 a muscle injury of the soleus muscle and induced an increased muscle force and functional improvement after transplantation of 10^6 myoblasts into the site of injury. Furthermore, muscular regeneration was preceded after muscle injury and immediate application of myoblasts into the site of injury (Irintchev et al. 1997; Wernig et al. 2000). These promising results were quickly followed by clinical trials. In the early 1990s intramuscular injection of allogenic myoblasts was per formed on humans with Duchenne Muscular Dystrophy (Gussoni et al. 1992, 1997; Mendell et al. 1995). Unfortunately, the clinical benefit obtained from these studies was minimal, and research programs attempted to recognize the failures and pitfalls of these clinical trials. Major limitations

of previously mentioned attempts included insufficient cell distribution, immune rejection, and poor cell survival after cell transplantation. According to knowledge based on preliminary experimental results, only a small number of cells participated in muscle regeneration whereas the vast majority of the injected cells did not survive the transplantation (Farini et al. 2009). In virtue of current data, satisfactory myoblast transplantation requires an ample delivery system of cells to the injured or degenerated tissue as well as a sufficient immunosuppressive therapy.

Genetically modified cells for gene delivery into muscle: It has been suggested that overproduction of pleiotropic cytokines into the injured tissue via genetically modified cells may represent an attractive alternative to conventional therapeutic strategies. An interesting method, which has been already used, for tissues repair of muscle, skin, liver and cartilage is to transfect specific cells with genome which enhances repair processes. For this purpose transfection of myoblasts may be an interesting option since these cells are used to repair and regenerate damaged skeletal muscle by acting as vectors for gene therapy. Genetically modified myoblasts have been used for replacing degenerating muscle fibers in mdx mice. As a gene delivery vehicle, myoblasts were used to deliver growth hormones, VEGF, Factor IX, EPO, FGF and others. Recent findings predict that targeted delivery of mRNA or DNA into the site of injury or injured cells will specifically manipulate genes and enhance muscle regeneration (Järvinen et al. 2005; Caplan 2007; Krampera et al. 2007).

42.1.6 Scaffolds and Muscle Regeneration

Transplantation of cells or the use of growth factors is a suitable procedure to treat minor defects after muscle injury. The application of cell-containing-scaffolds into the site of injury shows advances compared to the previously mentioned methods particularly in the treatment of larger muscle defects. Detailed in-vitro studies have enabled the development of scaffolds with the ability to generate muscle tissue. Aim of experimental approaches with scaffolds is to restore the structure and function of the injured muscle without scar tissue formation (Grefte et al. 2007). Seeding of the matrix with autologous satellite cells reduces not only the inflammation but also decreases the fibrosis at the edge of the implant (Grefte et al. 2007). Moreover, growth factors like FGF-2 or HGF inside the matrix have a positive effect on local myoblasts and improve myogenesis (Hill et al. 2006a, b). Further studies have shown that the matrix in which the cells are embedded plays a major role in the regenerative process. The use of myoblasts in scaffolds containing polyglycolic acid meshes, alginate or hyaluronic acid constructs promotes the vascularization and muscle neoformation (Saxena et al. 1999, 2001; Kamelger et al. 2004; Stratos et al. 2011). Myoblast can furthermore fuse in-vitro and can develop physiological function like force production. For this purpose a three-dimensional matrix can be used, were myoblasts are seeded and differentiated on top of a fibrin gel (Huang et al. 2005).

42.1.7 Future Perspectives

Although major scientific efforts have been made to understand the principles of myogenesis and muscular regeneration, no definitive treatment for muscle injury and degenerative muscle disease exists. The primary focus of current studies has been to identify molecules and cascades that can regulate the proliferation of satellite cells, influence the tissue inflammation, control the angiogenesis and affect the apoptosis of skeletal muscle. Further studies are needed to define the specific role of stem cells, scaffolds and other growth factors in the regeneration after injury, degeneration and dystrophy. Further goal should be to identify molecules that can modulate muscle cell homeostasis as well as to be able to boost proliferation and cell survival. These results will enhance our understanding of cell biology and cell regeneration serving as a platform for patient oriented based therapies.

42.2 Regeneration of Ligaments

Ligaments are non-stretchable strings of the skeleton, they contain mainly collagen and connect bones together. Ligamental tissue consists of an extracellular matrix with its embedded fibroblasts. The fibroblasts are responsible for biologic adaption to the mechanical environment, remodeling and healing of the injured ligament. Immediately after ligament injury (phase I) bleeding of the injured tissue occurs. This phase is followed by an inflammatory response (phase II) as various cytokines and growth factors are released by the inflammatory cells. This results in neovascularization and initiation of granulation tissue formation. Later fibroblasts proliferate (phase III) and collagen is being formed. Remodeling (phase IV) occurs 6 or more weeks after injury as wound gap is being filled with unorganized granulation tissue. This phase of healing can extend up to many years and is responsible for restoration of tensile stiffness and strength (Woo et al. 2004).

42.2.1 Ligamentisation and Mechanical Load

A common surgical procedure in humans for ligament replacement is to use autologous tendon grafts. The biological and morphological changes which take place after tendon transplantation are defined as ‘ligamentisation’ (Amiel et al. 1986). During the first two months a fibroblast proliferation occurs followed by graft remodeling, angiogenesis, vascularization and necrosis. Throughout the steady maturation of the graft and within 3 years after transplantation the tissue undergoes a complete metaplasia to a ligamentous structure.

Application of mechanical loading has been reported to positively influence the cellular proliferation as well as to effect cellular morphology and alignment in the regenerating ligament. Additionally, mechanical load influences the ligamental

regeneration by modulating the healing of the graft-bone interface. According to recent studies both the timing as well as the magnitude of mechanical stimulation after ligament injury are important for the optimal healing during the regeneration period (Rodeo et al. 2010).

42.2.2 Cell Therapy and Scaffolds

In-vivo studies have shown ligament regeneration by implanting mesenchymal stem cells and silk scaffold (Fan et al. 2008, 2009) resulting in a histological and functional improvement. Current in-vitro and in-vivo experiments suggest that subsequent to transplantation of autologous or allogenic mesenchymal stem cells, the transplanted cells display phenotypic characteristics of the endogenous surrounding tissue. These studies suggested that administration of mesenchymal stem cells at the site of injury reduces the injury size and enhances the regeneration (Arthur et al. 2009).

Clinical trials as well animal studies have pointed out the efficacy of platelet-rich plasma treatment for ligament and tendon injuries (Rodeo et al. 2010; Paoloni et al. 2011). Platelet-rich plasma is produced after centrifugation of whole blood. That centrifugate holds not only higher platelet concentration than that of the whole blood but contains also numerous growth factors that can participate into regeneration after injury. However, the efficacy of such treatment remains controversially discussed especially when comparing platelet-rich plasma treated subjects with corresponding sham groups (Paoloni et al. 2011).

Further scientific efforts have been made to generate adequate ligament scaffolds. The scaffolds should be biodegradable, porous, biocompatible, exhibit sufficient mechanical strength, and promote the formation of ligamentous tissue (Cooper et al. 2005). In summary, collagen fiber scaffolds as well as hybrid biomaterial-biological ensembles have been proposed to enhance ligament regeneration after injury (Kew et al. 2011). Current literature distinguishes between biologic and synthetic scaffolds, however both of them seem to exhibit inadequate tensile strength as well as to have fatique properties (Ignatius and Durselen 2009).

42.2.3 Matrix Metalloproteinases, Ultrasound Application and Nitric Oxide (NO)

Recently, extracellular proteins were identified to modulate ligament regeneration. The metalloproteinases are proteins, which function, in both extracellular environment through transmembrane and intracytoplasmic domains. Matrix metalloproteinases induce further production of pro-inflammatory cytokines and are counterbalanced by the Tissue Inhibitors of Metalloproteinases (TIMPs). The major role of the matrix metalloproteinases is to contribute to normal tissue remodeling mainly through breaking down any extracellular matrix component. Furthermore low-intensity ultrasound has been shown to have angiogenic actions on the ligaments as well as

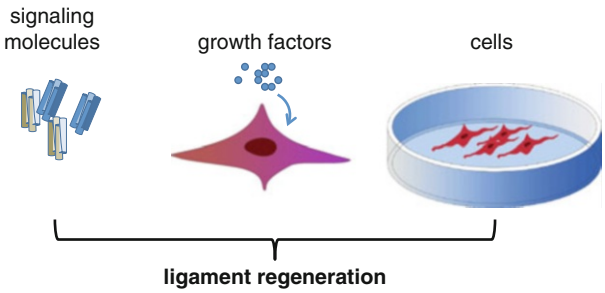


Fig. 42.2 A proposed approach achieving successful ligament regeneration combining signaling molecules, growth factors and cell transplantation

gene expression and proteoglycan synthesis. The NO is a free radical agent which acts in intracellular and extracellular environment. NO is enhanced during ligament healing (Deehan and Cawston 2005).

42.2.4 Growth Factors and Gene Therapy by Ligament Regeneration

A variety of growth factors have been identified to modulate the regenerative capacity of ligaments and to improve tissue function. Researchers have tried to develop strategies that improve ligament regeneration and repair mainly by modifying the extent of scar tissue formation due to the fact that ligament regeneration is similar to the healing process of the skin. Platelet-derived growth factor (Hildebrand et al. 1998), epidermal growth factor and transforming growth factor as well as the fibroblast growth factor (DesRosiers et al. 1996; Deehan and Cawston 2005), promotes the angiogenesis and scar tissue formation of the ligament. Growth factors can be applied to modulate many cellular activities, including cell proliferation, cell migration, and extracellular matrix synthesis and production.

Gene therapy introduces foreign nucleic acids into cells in order to alter their endogenous protein expression. Direct transfer involves the use of naked DNA from mammalian tissue and a one-step delivery of genes into host cells in vivo. Indirect transfer involves transfection of desired genes into cells followed by implantation of the cellular tissue into the host. By means of tissue engineering it is possible to transfect cells with beneficial factors and to inject them into the injured ligament (Menetrey et al. 1999). This results in an improved ligament healing and a faster maturation. One of the major drawbacks of this technique is the mutagenesis, which is clinically unacceptable especially in elective cases.

It is clear from the current literature, that regeneration of ligaments is possible and that growth factors, cell transplantation as well as scaffolds (Fig. 42.2) do have a capacity to repair. Unfortunately none of the previously mentioned methods has

been analyzed clinically. Considering the limited number of clinical and experimental studies as well as our poor knowledge regarding ligament regeneration, we conclude that at present tissue engineering of ligaments only partially fulfills scientist's expectations due to the challenge of achieving a sufficient primary tensile strength and adequate tissue angiogenesis.

References

Amiel D, Kleiner JB, Roux RD et al (1986) The phenomenon of "ligamentization": anterior cruciate ligament reconstruction with autogenous patellar tendon. J Orthop Res 4:162–172

Arai C, Ohnuki Y, Umeki D et al (2006) Effects of clenbuterol and cyclosporin A on the myosin heavy chain mRNA level and the muscle mass in rat masseter. J Physiol Sci 56:205–209

Armour J, Tyml K, Lidington D et al (2001) Ascorbate prevents microvascular dysfunction in the skeletal muscle of the septic rat. J Appl Physiol 90:795–803

Arthur A, Zannettino A, Gronthos S (2009) The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair. J Cell Physiol 218:237–245

Barton ER, Morris L, Musaro A et al (2002) Muscle-specific expression of insulin-like growth factor I counters muscle decline in mdx mice. J Cell Biol 157:137–148

Baskin CR, Hinchcliff KW, DiSilvestro RA et al (2000) Effects of dietary antioxidant supplementation on oxidative damage and resistance to oxidative damage during prolonged exercise in sled dogs. Am J Vet Res 61:886–891

Bolcal C, Yildirim V, Doganci S et al (2007) Protective effects of antioxidant medications on limb ischemia reperfusion injury. J Surg Res 139:274–279

Caplan AI (2007) Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol 213:341–347

Charge SB, Rudnicki MA (2004) Cellular and molecular regulation of muscle regeneration. Physiol Rev 84:209–238

Ciciliot S, Schiaffino S (2009) Regeneration of mammalian skeletal muscle. Basic mechanisms and clinical implications. Curr Pharm Des 16(18):906–914

Cooper JA, Lu HH, Ko FK et al (2005) Fiber-based tissue-engineered scaffold for ligament replacement: design considerations and in vitro evaluation. Biomaterials 26:1523–1532

Corbel SY, Lee A, Yi L et al (2003) Contribution of hematopoietic stem cells to skeletal muscle. Nat Med 9:1528–1532

De Bari C, Dell'Accio F, Vandenabeele F et al (2003) Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. J Cell Biol 160:909–918

Deehan DJ, Cawston TE (2005) The biology of integration of the anterior cruciate ligament. J Bone Joint Surg Br 87:889–895

DesRosiers EA, Yahia L, Rivard CH (1996) Proliferative and matrix synthesis response of canine anterior cruciate ligament fibroblasts submitted to combined growth factors. J Orthop Res 14:200–208

Dezawa M, Ishikawa H, Itokazu Y et al (2005) Bone marrow stromal cells generate muscle cells and repair muscle degeneration. Science 309:314–317

Erkanli K, Kayalar N, Erkanli G et al (2005) Melatonin protects against ischemia/reperfusion injury in skeletal muscle. J Pineal Res 39:238–242

Evans WJ (2000) Vitamin E, vitamin C, and exercise. Am J Clin Nutr 72:647S–652S

Fan H, Liu H, Wong EJ et al (2008) In vivo study of anterior cruciate ligament regeneration using mesenchymal stem cells and silk scaffold. Biomaterials 29:3324–3337

Fan H, Liu H, Toh SL et al (2009) Anterior cruciate ligament regeneration using mesenchymal stem cells and silk scaffold in large animal model. Biomaterials 30:4967–4977

Farini A, Razini P, Erratico S et al (2009) Cell based therapy for Duchenne muscular dystrophy. J Cell Physiol 221:526–534
Ferrari G, Cusella-De Angelis G, Coletta M et al (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279:1528–1530
Galli R, Pagano SF, Gritti A et al (2000) Regulation of neuronal differentiation in human CNS stem cell progeny by leukemia inhibitory factor. Dev Neurosci 22:86–95
Grefte S, Kuijpers-Jagtman AM, Torensma R et al (2007) Skeletal muscle development and regeneration. Stem Cells Dev 16:857–868
Gussoni E, Pavlath GK, Lanctot AM et al (1992) Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation. Nature 356:435–438
Gussoni E, Blau HM, Kunkel LM (1997) The fate of individual myoblasts after transplantation into muscles of DMD patients. Nat Med 3:970–977
Halici M, Narin F, Turk CY et al (2004) The effect of melatonin on plasma oxidant-antioxidant skeletal muscle reperfusion injury in rats. J Int Med Res 32:500–506
Hildebrand KA, Woo SL, Smith DW et al (1998) The effects of platelet-derived growth factor-BB on healing of the rabbit medial collateral ligament. An in vivo study. Am J Sports Med 26:549–554
Hill E, Boontheekul T, Mooney DJ (2006a) Designing scaffolds to enhance transplanted myoblast survival and migration. Tissue Eng 12:1295–1304
Hill E, Boontheekul T, Mooney DJ (2006b) Regulating activation of transplanted cells controls tissue regeneration. Proc Natl Acad Sci USA 103:2494–2499
Hu J, Du J, Zhang L et al (2010) XIAP reduces muscle proteolysis induced by CKD. J Am Soc Nephrol 21:1174–1183
Huang YC, Dennis RG, Larkin L et al (2005) Rapid formation of functional muscle in vitro using fibrin gels. J Appl Physiol 98:706–713
Husmann I, Soulet L, Gautron J et al (1996) Growth factors in skeletal muscle regeneration. Cytokine Growth Factor Rev 7:249–258
Ignatius A, Durselen L (2009) Possibilities and limits in tissue engineering of the anterior cruciate ligament. Orthopade 38:1080–1086
Irintchev A, Langer M, Zweyer M et al (1997) Functional improvement of damaged adult mouse muscle by implantation of primary myoblasts. J Physiol 500(Pt 3):775–785
Israeli D, Benchaouir R, Ziaei S et al (2004) FGF6 mediated expansion of a resident subset of cells with SP phenotype in the C2C12 myogenic line. J Cell Physiol 201:409–419
Jackman RW, Kandarian SC (2004) The molecular basis of skeletal muscle atrophy. Am J Physiol Cell Physiol 287:C834–C843
Järvinen TA, Järvinen TL, Kääriäinen M et al (2005) Muscle injuries: biology and treatment. Am J Sports Med 33:745–764
Jiang Y, Vaessen B, Lenvik T et al (2002) Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol 30:896–904
Kamelger FS, Marksteiner R, Margreiter E et al (2004) A comparative study of three different biomaterials in the engineering of skeletal muscle using a rat animal model. Biomaterials 25:1649–1655
Karalaki M, Fili S, Philippou A et al (2009) Muscle regeneration: cellular and molecular events. In Vivo 23:779–796
Kearns SR, Daly AF, Sheehan K et al (2004) Oral vitamin C reduces the injury to skeletal muscle caused by compartment syndrome. J Bone Joint Surg Br 86:906–911
Kew SJ, Gwynne JH, Enea D et al (2011) Regeneration and repair of tendon and ligament tissue using collagen fibre biomaterials. Acta Biomater 7:3237–3247
Kim EK, Hong JP (2007) The effect of recombinant human erythropoietin on ischemia-reperfusion injury: an experimental study in a rat TRAM flap model. Plast Reconstr Surg 120:1774–1781
Kon M, Kimura F, Akimoto T et al (2007) Effect of Coenzyme Q10 supplementation on exercise-induced muscular injury of rats. Exerc Immunol Rev 13:76–88

Krampera M, Franchini M, Pizzolo G et al (2007) Mesenchymal stem cells: from biology to clinical use. Blood Transfus 5:120–129
LaBarge MA, Blau HM (2002) Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. Cell 111:589–601
Li J, Yang L, Liu K et al (2010) Synergistic effects of FGF-2 and PDGF-BB on angiogenesis and muscle regeneration in rabbit hindlimb ischemia model. Microvasc Res 80(1):10–17
Mauro A (1961) Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 9:493–495
McKay BR, O'Reilly CE, Phillips SM et al (2008) Co-expression of IGF-1 family members with myogenic regulatory factors following acute damaging muscle-lengthening contractions in humans. J Physiol 586:5549–5560
Mendell JR, Kissel JT, Amato AA et al (1995) Myoblast transfer in the treatment of Duchenne's muscular dystrophy. N Engl J Med 333:832–838
Menetrey J, Kasemkijwattana C, Day CS et al (1999) Direct-, fibroblast- and myoblast-mediated gene transfer to the anterior cruciate ligament. Tissue Eng 5:435–442
Mitchell PO, Pavlath GK (2002) Multiple roles of calcineurin in skeletal muscle growth. Clin Orthop Relat Res 403:S197–S202
Motohashi N, Uezumi A, Yada E et al (2008) Muscle CD31(−) CD45(−) side population cells promote muscle regeneration by stimulating proliferation and migration of myoblasts. Am J Pathol 173:781–791
Mourkioti F, Rosenthal N (2008) NF-kappaB signaling in skeletal muscle: prospects for intervention in muscle diseases. J Mol Med 86:747–759
Muguruma Y, Reyes M, Nakamura Y et al (2003) In vivo and in vitro differentiation of myocytes from human bone marrow-derived multipotent progenitor cells. Exp Hematol 31:1323–1330
Naito T, Goto K, Morioka S et al (2009) Administration of granulocyte colony-stimulating factor facilitates the regenerative process of injured mice skeletal muscle via the activation of Akt/GSK3alphabeta signals. Eur J Appl Physiol 105:643–651
Oestern HJ, Tscherne H (1983) Physiopathology and classification of soft tissue lesion. Hefte Unfallheilkd 162:1–10
Otto A, Collins-Hooper H, Patel K (2009) The origin, molecular regulation and therapeutic potential of myogenic stem cell populations. J Anat 215:477–497
Paoloni J, De Vos RJ, Hamilton B et al (2011) Platelet-rich plasma treatment for ligament and tendon injuries. Clin J Sport Med 21:37–45
Peterson JM, Guttridge DC (2008) Skeletal muscle diseases, inflammation, and NF-kappaB signaling: insights and opportunities for therapeutic intervention. Int Rev Immunol 27:375–387
Price FD, Kuroda K, Rudnicki MA (2007) Stem cell based therapies to treat muscular dystrophy. Biochim Biophys Acta 1772:272–283
Qu Z, Balkir L, van Deutekom JC et al (1998) Development of approaches to improve cell survival in myoblast transfer therapy. J Cell Biol 142:1257–1267
Reznick AZ, Witt E, Matsumoto M et al (1992) Vitamin E inhibits protein oxidation in skeletal muscle of resting and exercised rats. Biochem Biophys Res Commun 189:801–806
Rodeo SA, Delos D, Weber A et al (2010) What's new in orthopaedic research. J Bone Joint Surg Am 92:2491–2501
Rodriguez AM, Pisani D, Dechesne CA et al (2005) Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. J Exp Med 201:1397–1405
Rotter R, Menshykova M, Winkler T et al (2008) Erythropoietin improves functional and histological recovery of traumatized skeletal muscle tissue. J Orthop Res 26:1618–1626
Saxena AK, Marler J, Benvenuto M et al (1999) Skeletal muscle tissue engineering using isolated myoblasts on synthetic biodegradable polymers: preliminary studies. Tissue Eng 5:525–532
Saxena AK, Willital GH, Vacanti JP (2001) Vascularized three-dimensional skeletal muscle tissue-engineering. Biomed Mater Eng 11:275–281
Sherwood RI, Christensen JL, Weissman IL et al (2004) Determinants of skeletal muscle contributions from circulating cells, bone marrow cells, and hematopoietic stem cells. Stem Cells 22:1292–1304

Singleton JR, Feldman EL (2001) Insulin-like growth factor-I in muscle metabolism and myotherapies. Neurobiol Dis 8:541–554
Stratos I, Rotter R, Eipel C et al (2007) Granulocyte-colony stimulating factor enhances muscle proliferation and strength following skeletal muscle injury in rats. J Appl Physiol 103:1857–1863
Stratos I, Madry H, Rotter R et al (2011) Fibroblast growth factor-2-overexpressing myoblasts encapsulated in alginate spheres increase proliferation, reduce apoptosis, induce adipogenesis, and enhance regeneration following skeletal muscle injury in rats. Tissue Eng Part A 17:2867–2877
Stratos I, Li Z, Rotter R et al (2012a) Inhibition of caspase mediated apoptosis restores muscle function after crush injury in rat skeletal muscle. Apoptosis 17(3):269–277
Stratos I, Richter N, Rotter R et al (2012b) Melatonin restores muscle regeneration and enhances muscle function after crush injury in rats. J Pineal Res 52:62–70
Takahashi T, Kalka C, Masuda H et al (1999) Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 5:434–438
Torrente Y, Belicchi M, Sampaolesi M et al (2004) Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. J Clin Invest 114:182–195
Wagner KR (2005) Muscle regeneration through myostatin inhibition. Curr Opin Rheumatol 17:720–724
Warren JA, Jenkins RR, Packer L et al (1992) Elevated muscle vitamin E does not attenuate eccentric exercise-induced muscle injury. J Appl Physiol 72:2168–2175
Wernig A, Zweyer M, Irintchev A (2000) Function of skeletal muscle tissue formed after myoblast transplantation into irradiated mouse muscles. J Physiol 522(Pt 2):333–345
Woo SL, Jia F, Zou L et al (2004) Functional tissue engineering for ligament healing: potential of antisense gene therapy. Ann Biomed Eng 32:342–351
Yimlamai T, Dodd SL, Borst SE et al (2005) Clenbuterol induces muscle-specific attenuation of atrophy through effects on the ubiquitin-proteasome pathway. J Appl Physiol 99:71–80
You T, Goldfarb AH, Bloomer RJ et al (2005) Oxidative stress response in normal and antioxidant supplemented rats to a downhill run: changes in blood and skeletal muscles. Can J Appl Physiol 30:677–689

Chapter 43
Skin

Hans-Günther Machens, Christina Irene Günter, and Augustinus Bader

Abstract Skin is the largest organ in the human body. Its surface ranges in average between 1.5 and 1.8 m^2 and the thickness varies between 0.5 (lower eyelid) and 15 mm (foot sole) in a young healthy adult, resulting in a tissue volume of 7,500–27,000 mm^3. The skin has to fulfill a magnitude of physiological organic tasks, which is indicated by the variety of tissue thicknesses. These tasks include mechanistic, metabolic, energetic and immunologic aspects. Skin was the first organ which had been tissue engineered in vitro and translated back into clinical application. Therefore it is a prime target for regenerative therapies, not only due to its easy accessibility but also, because of the fact that skin is one of the most active and continuously regenerating organs and therefore a fascinating model to learn more about the human body's intrinsic regenerative mechanisms.

This book chapter focuses on the regenerative capacities of skin tissue and its comprising cell compartments and explains how the principles of skin regeneration may be translated into clinical practice.

H.-G. Machens, M.D. (✉)
Clinic for Plastic and Hand Surgery, University Hospital Rechts der Isar, Technische Universitaet Muenchen, Ismaninger Str. 22, 81675 Munich, Germany
e-mail: Machens@lrz.tu-muenchen.de

C.I. Günter, M.D.
Clinical Trials Research Group, Clinic for Plastic and Hand Surgery, University Hospital Rechts der Isar, Technische Universität München, Ismaninger Str. 22, 81675 Munich, Germany

A. Bader, M.D.
Stem Cell Biology and Cell Technology, Medical Faculty, Biotechnological-Biomedical Center (BBZ), Leipzig University, Deutscher Platz 5, D-04103 Leipzig, Germany

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_43,

Abbreviations

TEN	Toxic epidermal necrolysis
EPO	Erythropoietin
EPOR2	EPO Receptor 2
EPOβ 1/2	EPO Receptor β 1 or 2
TNF-α	Tumour Necrosis Factor α
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8
TGFβ 1–3	Transforming growth factor beta 1–3
PDGF	Platelet-derived growth factor
PLC	Phospholipase C
PKB	Proteinkinase B
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
BAD	Bcl-2-Antagonist of Cell Death
GSK-3β	Glycogen Synthase Kinase 3
NO	Nitric oxide
Ca	Calcium

43.1 Introduction

Skin is the natural barrier between the human body and its environment. Therefore, it is frequently challenged by extrinsic noxes of any kind, but also intrinsic factors may be cause for damage to the skin (e.g. auto immunologic noxes). Due to permanent loss of tissue and cell fractions within its surface, skin is continuously in the process of regeneration. It is the only organ which enables even the medically untrained to give an accurate diagnosis about its morphological and functional status (e.g. aging). But what is the cellular and molecular motor behind the fascinating regenerative capacities of skin tissue? The following book chapters will give insight into the developmental and structural principles of skin, its repair and regeneration tools after damage and the resulting therapeutic modalities, which could be the result of a better understanding of skin regeneration.

43.2 Skin Development and Stem Cell Function

Dermis and epidermis divide the skin tissue structurally into two major components. The superficial epidermis consists mainly of keratinocytes in different developmental stages and represents the most superficial layer of the skin. It is a squamous epithelium with several strata: the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale (Freedberg et al. 2003). The dermal

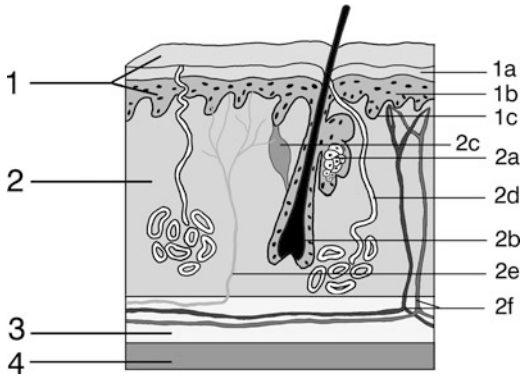


Fig. 43.1 Anatomical structure of the human skin. *1* Epidermis: *a* Stratum corneum; *b* Stratum germinativum, *c* Basement membrane. *2* Dermis: *2a* Sebaceous gland, *2b* Hair follicle, *2c* Arrector pili muscle, *2d* Sweat gland, *2e* Nerve, *2f* Capillaries and vascular plexus. *3* Subcutaneous fatty tissue. *4* Musculature

part is situated deeper in the body, below the epidermis. It represents the functionally important tissue and is mainly comprised of matrix collagen type I, elastin, fibroblasts and skin appendices (capillary, sweat and sebaceous glands, sensory corpuscles, blood vessels). Cellular nutrition to the layers of the epidermis is provided via diffusion from the dermis, since the epidermis has no direct blood supply. The epidermis consists of four cell types: keratinocytes, melanocytes, Langerhans cells, and Mekel cells. Of these, keratinocytes amount to 95% of the cells of the epidermis (Burns et al. 2006). This stratified squamous epithelium is maintained by constant cell division within the stratum basale, in which differentiating cells slowly migrate outwards through the stratum spinosum to the stratum corneum, where cornified cells are continually shed from the surface. In normal skin, the rate of reproduction equals the rate of loss; it takes an average of 2 weeks for a cell to migrate from the basal cell layer to the top of the granular cell layer, and an additional 2 weeks to cross the stratum corneum (Bolognia et al. 2007).

The dermis is the layer of skin between the epidermis and subcutaneous tissue, and is composed of two sections, the papillary and reticular dermis. The superficial papillary dermis interdigitates with the overlying rete ridges of the epidermis, the two layers interact through the basement membrane (Rapini 2005). Structural components of the dermis are collagen, elastic fibers, and extracellular and extrafibrillar matrix. Within these components are the pilosebaceous units, arrector pili muscles, and the eccrine and apocrine glands. The dermis contains two vascular networks that run parallel to the skin surface—one superficial and one deep plexus—which are connected by vertical communicating vessels (Freedberg et al. 2003). The function of blood vessels within the dermis is twofold: to supply nutrition and to regulate temperature. These blood vessels are also crucial for regaining rapid reconnection with underlying blood vessels after split skin transplantation on wounds by a process called ‘inosculation’ within 24 h after transplantation (Converse et al. 1975) (Fig. 43.1 and Table 43.1).

Skin tissue covers the surface of the embryo right from the beginning of the earliest embryologic stages and has contributions from two germ layers: Ectoderm forms the surface epidermis and the associated glands, whereas mesoderm forms the

Table 43.1 Skin: anatomical structure and function

Layer	Structure	Function
Epidermis	Epithelial cells	Barrier, protect against injury, contamination and moisture loss
	Melanocytes	Protect against UV-light, origin for skin pigmentation and tanning
	Langerhans cells	Antigen presenting, immuncompetent cells
Dermis	Collagen	Strength and support
	Elastin	Elasticity
	Nerves	Sensors for: pain, temperature, touch, vibration
	Capillaries	Nutrition and oxygen supply, waste removal, thermoregulation
	Fibroblasts	Mesenchymal derived cells producing the extra cellular matrix
Epidermal appendages	Hair follicles	Produce epidermis and hair, epidermis regeneration
	Sweat glands	Produce sweat: thermoregulation
	Sebaceous glands	Produce sebum: antimicrobial, maintains: pH, skin and hair condition
Subcutaneous tissue	Fat cells	Isolation, energy storage
	Stem cells	Pluripotent cells in the fat tissue enabling regeneration
	Connective tissue	Attaches skin, divides tissue into compartments

underlying connective tissue of the dermis. Ectodermally derived neural crest cells also migrate into the forming epidermis to populate with melanocytes and specialized sensory endings. For more detailed information we advice a multitude of detailed developmental overviews (McGrath et al. 1971) (Fig. 43.2).

Already during early embryologic development the ectodermal sheath becomes intrinsically important since it provides environmental protection already in the early gestational weeks. The further development into epidermal and dermal layers arises at a much later point of time, but the immanent stem cell population remains active within the later formation of the hair bulges. From here, pre-keratinocytes grow out and form the epidermal layers, losing subsequently its differentiating capacity while slowly migrating from the lower, more undifferentiated towards the outer, more differentiated cell layers and finally into the squamous epithelium.

It has been shown recently that both epidermally and dermally derived stem cells can differentiate into structures of all three germ layers, indicating the intrinsic potential of these cell sources within the skin (Rolletschek and Wobus 2009). Meanwhile laboratory protocols are described for isolation and cultivation of human keratinocytes from skin or plucked hair for the generation of induced pluripotent stem cells (Aasen and Belmonte 2010). A further resource of stem cells is located within the dermal layer, where fibroblasts present the main population of resident cells (Fernandes et al. 2008). Recent reports lead to the assumption that pluripotent stem cells can be found within this fibroblast cell population of the dermis (Lorenz et al. 2008)

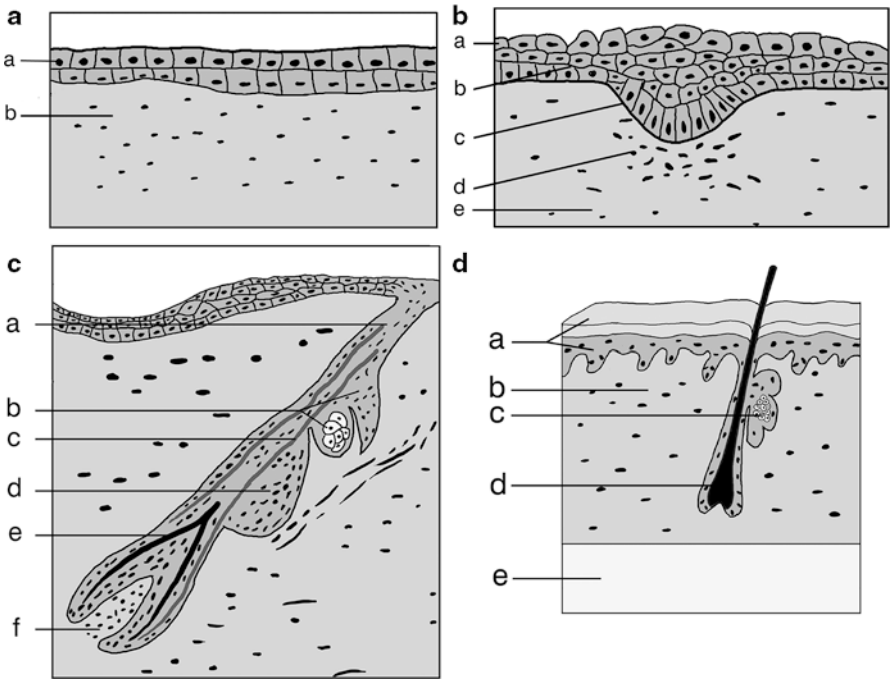


Fig. 43.2 Different embryological phases of skin development. (**a**) 4 weeks: *a* Surface ectoderm, *b* Mesenchym. (**b**) 7 weeks: *a* Periderm, *b* Germinal layer, *c* Hair germ, *d* Mesenchyme cells, *e* Mesenchyme. (**c**) 16 weeks: *a* Hair canal, *b* Sebaceous gland, *c* Inner root sheath, *d* Bulge, *e* Hair, *f* Mesenchyme cells. (**d**) Birth: *a* Epidermis, *b* Dermis, *c* Sebaceous gland, *d* Hair bulb, *e* Subcutaneous fatty tissue

Pluripotent stem cells can be recruited from glandular cells including sweat glands (Petschnik et al. 2009) pancreas and submandibulary gland (Egana et al. 2009)

Therefore, it seems worthwhile to take a closer look into the relation between skin development and stem cell function (Fig. 43.3).

Stem cells play a crucial role in postnatal skin maintenance, since they provide permanent recruitment of important functional tissue in the dermal and epidermal compartment. Within the different stem cell compartments, epidermal stem cells may even play an exceptional role: since the epidermis continually renews itself by sloughing a layer of cells every day, it is in a constant state of cellular turnover and requires continual cell replacement for life. Thus, maintaining a vital epidermal stem cell population is of prime importance, even during aging. Unlike stem cells from internal tissues, epidermal stem cells show little response to aging (Racila and Bickenbach 2009). They do not appear to decrease in number or functionality with age, and do not show changes in gene expression, developmental responsiveness, or age-associated increases of reactive oxygen species. While human skin grows older, the stem cells within have no loss in numbers and in their regenerative capacity. It is tempting to hypothesize that the process of aging is strongly related to stem cell reservoir and functional capacity in the dermal layer of the skin.

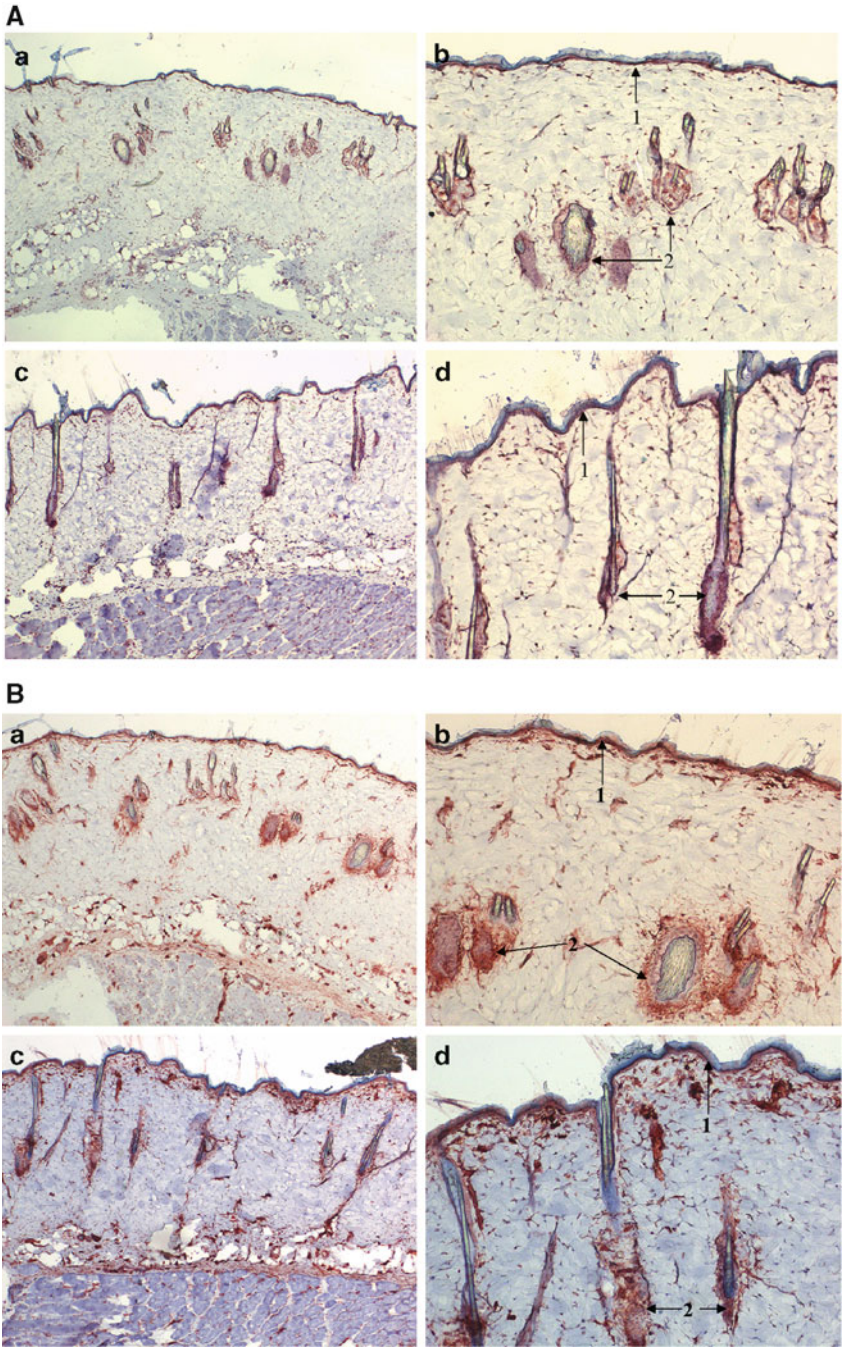


Fig. 43.3 Stem cell participation in postnatal skin structure. (**a**) Immunhistological images of rat skin tissue sections, stained with monoclonal antibody against nestin for identification of neuronal stem cells, **A/C** magnification × 40, **B/D** magnification × 100; *1* basement membrane (basal lamina); *2* hair follicle. (**b**) Immunhistological images of rat skin tissue sections, stained with antibody against CD 90 for identification of mesenchymal stem cells and fibroblasts, **A/C** magnification × 40, **B/D** magnification × 100; *1* basement membrane (basal lamina); *2* hair follicle

43.3 Regenerative Repair Mechanisms (Trauma – Dependent Activation of ß1-EPO Receptors)

In recent years a new player in wound regeneration has been explored and described. It is a well known protagonist, which is used for the treatment of anaemia for more than two decades: Erythropoietin (EPO). Therefore potential risks and benefits of this innovative therapeutical tool can easily be estimate.

The tissue-protective effects of EPO seem to be mediated by a special EPO receptor-sub-type, which is postulated to be different from the EPOR2 of the erythropoietic system (Brines et al. 2004).

Signalling via the β1-EPO receptor initiates multiple, coordinated functions that counteract the stereotype injury response which occurs after any trauma and the possible further collateral damages. The organism reacts in this stereotypic manner to primarily prevent the invasion of pathogens and generalised infection; it does not differentiate between sterile and infectious pathogens, or between external trauma and internal stress reactions (Lotze et al. 2007).

Initially, the reaction is mediated via members of the pro-inflammatory Type I cytokine family such as TNF-α (Takeuchi et al. 2007). In turn multiple players like 'free radicals' and other highly reactive molecules are released. Herewith, pathogens which potentially have penetrated the organism are destroyed, whereby healthy cells are destroyed as well (collateral damage) (Brines et al. 2008).

The inhibition of the primary injury response is of utmost importance for a normal wound healing process. EPO and TNF-α inhibit their mutual production and biological activity (Bernaudin et al. 1999); additionally EPO receptor expression is enhanced by TNF-α, creating a balance between the initial injury response and the inhibitory EPO system.

In the following regenerative wound healing steps, EPO recruits stem cells and thus ensures that they are present in the area of regeneration. Additionally it also triggers the release of specific growth factors (Viviani et al. 2005).

An acute tissue protective action of EPO is the iNOS and eNOS triggered vasodilatation shortly after trauma, resulting in a persisting blood supply of the surrounding tissues (Rezaeian et al. 2008, 2010).

Another pro-regenerative EPO action is the protection of capillary endothelial cells which, under hypoxic stress would become apoptotic, thus providing improved blood supply to surrounding areas (Peterson et al. 2007).

In addition, an inhibitory influence of EPO is impacted upon specialised cells for the prevention of infection, such as leukocytes and macrophages: thus the production of pro-infective interleukins and interferons as well as TNF-α is inhibited (Schultz et al. 2008; Yazihan et al. 2008).

Acknowledgment: Thanks for excellent contribution in preparing the histological specimens with great acknowledgment to Sabine Ebert from University of Leipzig, Centre for Biotechnology and Biomedicine, Department of Applied Stem Cell Biology and Cell Techniques, Germany

EPO and EPO receptors are reported to be produced in the skin, where in addition the typical cell protective effects could be demonstrated. The same has been examined for hair follicles; here a protective effect against chemotherapy-induced apoptosis of hair follicle cells could be revealed (Bodó et al. 2007).

Just recently our comprehension regarding the inaction between multiple players in skin regeneration was significantly improved. It could be demonstrated, that the regulation of mesenchymal stem cell growth is closely connected to the co-expression of EPO and several trauma cytokines such as Il-6 and TNF-α (Bader and Machens 2010).

43.4 Skin Diseases

The number of endo- and exogenous noxes, affecting the skin in its different cellular components and as a whole organ is countless. Certainly, thermal injury is the most devastating traumatic cause for total loss of all skin components at a single and exogenious incident. While auto immunologic agents, as endogenous factors, mostly affect the epidermal part or the epidermo-dermal junction (e.g. epidermolysis bullosa, toxic epidermal necrolysis = TEN).

As long as at least parts of skin tissue survives, regenerative tools are recruited to commence repair and regeneration. In general, it may be stated that the regenerative capacity of skin directly depends on three factors: the amount of surviving epidermo-dermal tissue, the regenerative capacity of local tissue in the trauma zone and ability of the organism to recruit new cells from other restorative compartments (e.g. bone marrow). In principle, loss of epidermis may be fully compensated and restitutio ad integrum achieved, as long as the dermis and its regenerative cellular departments are still intact and vital enough to reproduce the necessary cells and tissues (Table 43.2) .

43.5 Clinical Principles, Diagnostics, Indications of Skin Regenerative Therapies

Skin may regenerate even after total loss of the epidermo-dermal junction, depending on the regenerative capacity of the underlying tissue and the whole organism. It is sometimes clinically difficult to estimate the correct amount of surviving tissue, especially after partial loss of dermal tissue. In such instances, younger patients develop more regenerative activity as compared to elderly. Therefore, dermal hypertrophy can result on the one hand and total loss of remaining dermal tissue on the other. Clinical experience with these defects, e.g. thermally injured patients allows a more accurate estimation of wound depth and therefore prognosis of the patient (Figs. 43.4 and 43.5).

Table 43.2 Skin diseases, affected skin compartment and intrinsic regenerative tools

Disease	Affected compartiment	Regenerative tool	Healing	Scars
Epidermiolysis bullosa	Epidermo-dermal junction	Dermal stem cells	Spontaneous in 14–21 days	No
Toxic epidermal necrolysis	Epidermo-dermal junction	Dermal stem cells	Spontaneous in 14–21 days	No
Burn grade 1	Epidermal	Dermal stem cells of the stratum basale	Spontaneous in 5–10 days	No
Burn grade 2 a	Superficial dermal	Dermal stem cells in the stratum basale and the skin appendages	Spontaneous in 10–21 days	No
Burn grade 2 b	Deep dermal	Dermal stem cells in the skin appendages and remaining dermal components	Spontaneous in >21 days	Yes
Burn grade 3	Sub dermal	None	No spontaneous healing	Yes
Pressure sore	Sub dermal	None	No spontaneous healing	Yes

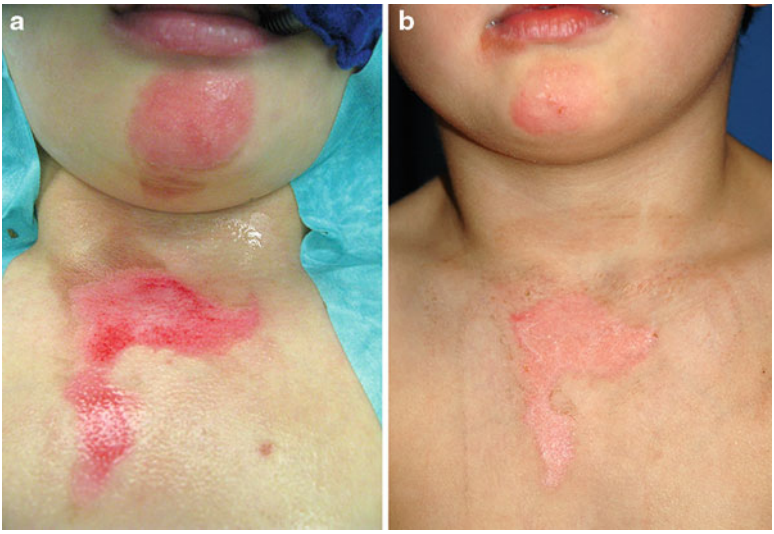


Fig. 43.4 (**a**) 2a Degree burn, superficial, 3 days after injury, careful cleaning and removing of the blisters in total anesthesia. (**b**) Spontaneous healing after 2 weeks. Total restitutio ad integrum was achieved

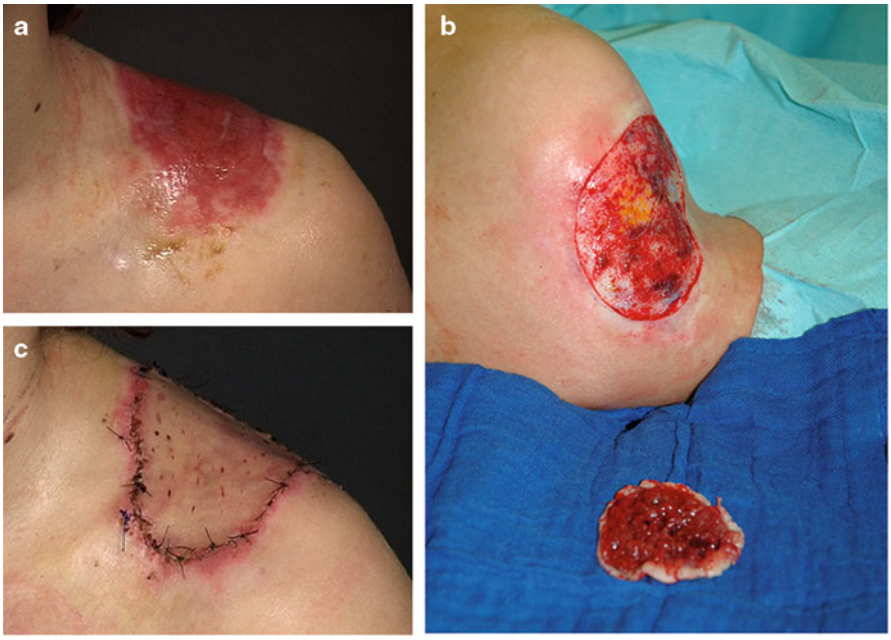


Fig. 43.5 (**a**) 2b Degree burn, non- healing, hypergranulatig wound after 3 weeks (**b**) The resulting granulation tissue was excised (**c**) and the defect transplated with an autologous split skin graft

A typical clinical example for a non-thermal 1st degree wound surface is TEN.

While the regeneration of epidermis is in process, the dermis and underlying tissue are prone to infection and injury. Therefore, it is mandatory to protect the integument both immunological and mechanically with a temporary epidermal skin substitute. Meanwhile, there is a variety of different industrial products available (e.g. Biobrane), which fulfill these requirements.

Partial loss of dermis may still be regenerated, as long as enough dermal tissue and especially the sub dermal vascular plexus are preserved. Typical examples are given by a 2a thermal injury and TEN, resulting in preservation of the deep dermal tissue. Clinically, after removal of bullae and blisters, a reddish wound bed appears. Recapillarisation occurs after gentle pressing on the wound surface, indicating an intact sub-dermal and partially intra-dermal vascular plexus with vital dermal capillaries. In this clinical picture, dermal regeneration can occur and sufficient nutrient and cellular supply transported into the wound area. The major complication in both, however, is systemic infection, especially in the elderly. The regenerative capacity becomes insufficient, when too much body surface is involved and not enough cellular repair and regeneration capacities are maintained.

If a full skin defect exists, split skin grafts, which contain dermal and epidermal parts, can be transplanted directly onto it, to enable a sufficient dermal-epidermal remodeling. But if the destroyed area of skin comprises more than 60% body surface area there are not sufficient amounts of autologous split-skin grafts to be taken, to cover the defect in one operation. Therefore, a variety of dermal substitutes or combined dermal and epidermal substitutes have been developed. They can be permanent or temporary, biological or synthetic or combined.

Xeno- and heterologic-split-skin-transplants and Epigard are temporary substitutes, which are subject to phagocytosis or have to be surgically removed. Therefore a second-step operation is necessary for permanent closure of the wound. Alloderm™, Biobrane™ and Integra™ need a second-step operation as well and a thin split-skin-graft transplantation as epidermal layer or a keratinocyte transplantation. However, previous to that, a neo-dermis has to develop by invasion of the collagen matrix via fibroblasts and angiogenesis must have occurred.

Cultured keratinocytes need, if transplanted as single cell layer or cell-suspension, to be able to develop an intact epidermis, an existing dermis, which ensures sufficient nutrient support and growth factor supply and other interactions such as formation of a stable epidermo-dermal junction. Otherwise these keratinocytes are prone to apoptosis or at least they do not continue to grow and do not form an epidermal layer. Thus it is not surprising that early attempts transplanting keratinocytes onto full thickness skin defects without a neo-dermis or dermis remnants allowed primary survival of severely burned patients but showed unsatisfying results after longer observation periods due to unstable wounds and secondary infection (Table 43.3).

Table 43.3 Gives an overview of some of the commonly used skin substitutes

Biological substitutes	Synthetic substitutes/Combined substitutes
Xeno-split-skin-transplants (porcine) temporary	Biobrane® (nylon, silicone, bovine collagen) permanent/temporary
Heterologic-split-skin-transplants (human) temporary	Integra® (shark hyaluronic acid, bovine collagen, silicone) permanent/temporary
Alloderm® (a-cellular, de-epidermalised human skin, with collagen and elastin) permanent	Epigard® (Gore-Tex, polyurethane) temporary
Autologus split skin grafts (autologous human) permanent	–
Amnion (heterologic or xenologic) temporary	–
Apligraf® (bovine collagen, heterologic fibroblasts and keratinocytes) temporary/ permanent	–
Dermagraf® (collagen-glycosamninoglycan matrix, heterologic fibroblasts) temporary/ permanent	–

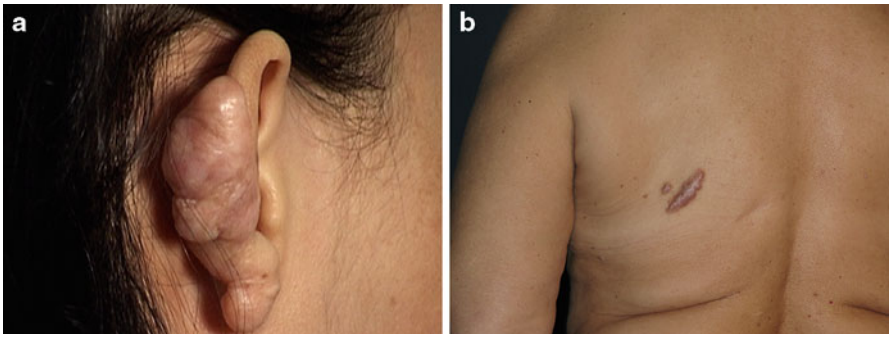


Fig. 43.6 (**a**) Keloid, (**b**) Hyperthropic scar

43.5.1 Hypertrophic and Keloid Scars

Scars develop always, if the cellular connections in the regenerative layers are divided and the dermis is completely interrupted. A typical clinical example is given after a clean cut with a sharp instrument like a scalpel for example. Scar formation also occurs after meticulous surgical repair. Interestingly, the tendency to form scar tissue is different in the human population and co depending on age, sex and race. Hypertrophic scarring and keloid formation are found more often in children and young adults than in the elderly. Persons who developed keloids in their childhood may not exhibit this tendency at an older age. Keloids and hypertrophic scars are more often found in more intensely pigmented skin than in less or no pigmented skin; this can be seen within one person as well as in different individuals. The incidence of keloid formation is 6–16% higher in darker pigmented populations as compared to Caucasians (See Fig. 43.6). Gender seems to be a risk factor too, as the female to male ratio is 2:1. Keloids can occur in every region of the body; they are

most commonly located on the upper trunk (chest, upper back, shoulders and ears). The etiology remains unclear. Hormone (estrogen) and growth factor influences (melanocyte stimulating hormone, MSH) may play a role, as well as different immunological influences and genetic predisposition. The diagnosis is mainly clinical: hypertrophic scars remain on the borders of the initial injury, whereas keloids grow over these boundaries like a pseudo tumor (Rockwell et al. 1989). Histologically, hypertrophic scars and keloids show both stretched and aligned collagen bundles, but fewer cells and capillaries are found in keloids. Today we know that although the macroscopic result of this scarring disorder cannot be seen for weeks or months, it is the result of very early dysregulation during wound healing (Meenakshi et al. 2005). Satisfactory, predictable and reliable therapies do not exist so far. Future therapies will have to be based on our improved understanding of regenerative mechanisms in the injured skin and will therefore lead to more specific therapeutic interventions.

43.6 Standardized Treatment and Technologies

43.6.1 Loss of Epidermis (1st Degree)

The classical example for a 1st degree thermal dermal injury is the sun-burn. Usually, as the dermis is intact in a first degree wound, it heals completely without scar formation within 4–8 days. It is moderately painful and in general there is usually no need for any specific analgetic therapy. There are abundant therapeutical options, most available products have a cooling and local analgetic effects, others just keep the wound moist.

43.6.2 Loss of Superficial Dermis (2a Degree)

Superficial second degree thermal dermal injuries usually result in blister formation. To prevent infections the blisters should be punctured carefully. As exposure of the open blister to air is extremely painful, blister removal should be considered carefully and only performed if necessary. Additionally an adequate analgetic therapy or especially in children a short anaesthesia should be taken into account.

There are two state of the art treatment options: the occlusive (removing the blisters and starting an occlusive local therapy) or the exposure (leaving the blisters intact as long as possible as a natural wound dressing) method. In Europe and Northern America the occlusive method is more commonly used. In less developed regions the exposure method is seen more frequently. It is a inexpensive, easy to perform method and as long as the scab is intact, the wound heals pain-free and very often without scar formation.

Concerning the occlusion method there exist a multitude of treatment options and products (silver nitrate, Marfenid, vinegar, iodine, silver sulfadiazine, etc.). Their most important common characteristics are the microbiological control and the preservation of a moist wound environment to enable undisturbed wound healing.

A broadly used product is Flammacine® (silver sulfadiazine), which is simple to handle and has a favourable cost-effectiveness ratio. Disadvantageous are the dressing changes which have to be performed at least daily and which are usually painful.

In our clinic our favourite occlusive method is the closure of the wound surface with synthetic membranes under strictly sterile conditions (for example Biobrane™) after careful surgical cleaning and debridement of the wound. These membranes stay in place until complete wound healing. This has the advantage that no dressing changes are necessary, although frequent wound controls have to be performed.

43.6.3 Loss of Deep Dermis (2b Degree)

After a deep second degree dermal thermal injury the necrotic superficial layers of the skin have to be removed surgically. Today's standard therapy is tangential necrectomy until sufficiently perfused layers are reached. This is easily recognised by little spot bleedings in the healthy dermis. After bleeding control, keratinocytes (as solution or as sheets) can be transplanted if enough dermal tissue is preserved or split skin grafts are used if deeper layers of dermis are involved. If after extensive thermal trauma the remaining non-damaged body surface does not allow for sufficient amounts of split skin grafts to be taken, temporary skin substitutes, such as heterologic or xeno split-skin grafts, amnion or Epigard can be used for a short period of time to prevent both infection and also hypertrophic granulation and later scar tissue formation.

In deep second degree injuries, the regenerative capacity of the skin will be exhausted in most of the cases. Without the transplantation of healthy split-skin grafts, the remaining dermal tissue will create a protective layer of granulation and later scar tissue as the most primitive, yet effective way to protect the wound from the environment and external noxes. It may be stated that in younger patients the regenerative capacity of mature and stem cells in this tissue compartment is stronger, compared to adults and elderly. Therefore, the production of scar tissue is also more rapid and pronounced. The therapeutic consequence in young patients is radical excision of the epidermo-dermal compartment down to the lowest dermal layers and split skin transplantation to prevent overgrafting.

43.6.4 Loss of Full Skin (3rd Degree)

When an acute full-thickness skin defect has occurred (for example 3rd degree burn) the wound has to be carefully cleaned and all remnants of necrotic skin have to be removed cautiously under surgical conditions. This means that the underlying tissue is also subjected to infection and trauma, since the biological barrier is lost.

If a clean wound bed is established, split skin grafts can be transplanted, or, after pre-treatment with a dermis substitute and neo-dermis formation, keratinocytes may be transplanted.

If a chronic full thickness skin defect exists (for example pressure sores, crural ulcer, and diabetic foot ulcers) the therapeutic strategy has to be different. That is due to the fact, that in chronic wounds an "anti-healing environment" prevails, with a majority of inhibitory factors which prevent healing. Additionally, the wound area is colonized with a multitude of microorganisms, which have to be at least grossly, most often surgically, eradicated before a definitive wound closure can be performed. If the wound is thus cleaned and necrotic tissue remnants have been removed, first and foremost the environment has to be changed from an anti-proliferate to a pro-proliferate environment.

Therefore, chronic inflammatory cascades have to be blocked. For example metalloproteinases and TNFα have to be antagonized and the concentrations of pro- proliferative factors such as EPO or TGFβ3 have to be increased. Then granulation tissue formation can successfully take place or a neo-dermis can be grown using a dermis substitute. Split skin grafts can then be transplanted on these prepared new wound bed if necessary.

If a pro-proliferative environment cannot be created due to advanced loss of vital and vascularised tissue, plastic surgical techniques have to be employed by using local or free tissue transfers to substitute the previous tissue loss in an adequate manner (Table 43.4).

43.7 Clinical Studies and Outcome of Skin Regenerative Therapies

43.7.1 Scar-Free Healing in the Embryo

A very interesting aspect is the scar-free healing of mammalian embryos, which came into the research focus just a few years ago. Several studies have been carried out to investigate adult and embryonic wound healing and scarring. In the meantime, most of the involved factors in adult and in embryonic skin regeneration are described. The most important factor seems to be the fact that in embryos the immune system and the inflammatory cascade are not sufficiently developed. Thus, the resulting inflammatory reaction in an embryo is much smaller and of a shorter time period than in more advanced developmental stages and adults. Additional key roles are played by TGFβ 1–3 and PDGF. If PDGF and TGFβ1 and 2 are neutralized and TGFβ3 is added to adult wounds, embryonic scar-free healing can be achieved. This was successfully demonstrated in rodents, pigs and healthy human volunteers. Following these promising results new scarring-preventing drugs are being developed and clinical trials are carried out (Ferguson et al. 2004). It could be shown that locally administered TGFbeta3 is well tolerated and improves skin regeneration and thus reduces scarring after trauma (Occleston et al. 2008).

Table 43.4 All four stages of skin loss

Grade	Anatomical layer	Appearance	Pain	Under-laying Tissue	Needle test	Recapillarisation	Healing	Scars
1	Epidermal	Red	Yes	Normal	Positive	Yes	5–10 days	No
2 a	Superficial dermal	Red ground bullae/ blisters	Yes	Oedema	Positive	Yes	2 weeks	No
2 b	Deep dermal	Big bullae and blisters Ground white or red	Moderate or none	Pronounced oedema	Delayed or negative	Delayed or none	>3 weeks	yes
3	Sub dermal	White or red	none	Dried out coriaceous	Negative	None	No spontaneous healing	yes

43.7.2 Clinical Studies

There are abundant studies on cosmetic skin alterations and their therapeutical options. Only in recent years the regenerative trigger for diseases with partial or total dermis loss has come into focus. Dermatological disorders of the skin have also been studied extensively in clinical prospective trials. Very few data, however, are available on life-threatening skin wounds. Mostly, trials on testing wound dressings after split-skin transplantation have been performed with little focus on skin regeneration. A major reason for the lack of evidence-based data in this field is probably the fact that each traumatic, thermally induced wound has its own special pattern and therefore is not standardized, which is a pre-reqisite for clinical trials. Full skin defects have also been treated with dermal substitutes during the last 30 years. Therefore several products have reached routine clinical practice.

However, there is a one exception from this problem: the surgically induced split-skin graft donor site, which is created by a surgical instrument (dermatome), thus exactly defining depths and size of the surgically created wound. This, in fact, is the only standardized traumatic wound in clinical practice. More than 50 studies have been carried out to compare different strategies of locally applied therapeutics, especially dressings. None of these, however, has focused on the biological regenerative effects on a cellular level. Primary treatment target was always the time needed until complete re-epithelialisation was achieved.

In a recent publication, a multi-layer tissue engineering approach to cover large full thickness defects was described. In this approach, keratinocyte and fibroblast primer cell cultures are established from autologous skin biopsies. Cells are grown on special hyaluronic acid matrices, with which they are transplanted in two-time, two-step operative procedures. If necessary, a thin split skin graft expanded to 1:6 can be added later on. That way, a completely autologous and biological fully active epidermal-dermal substitute is realized (Hollander 2004).

There are several publications investigating the effects of EPO on skin regeneration. But only two report about EPO treatment in humans.

In a full-thickness-defect mouse model treated with EPO, the healing process clearly improved in a dose dependent manner (Sorg et al. 2009). These pro-regenerative effects could be shown in acute and chronic, ischemic and diabetic environments (Galeano et al. 2004, 2006; Buemi et al. 2004).

In patients even sclerodermic ulcers improved statistically significant under EPO therapy (Ferri et al. 2007). Keast and Fraser reported about four paraplegic patients, whose decubital ulcers improved significantly under systemic EPO treatment (Keast and Fraser 2004).

At present, the first large, prospective, randomised, double-blind, multi-centre study, founded by the German federal ministry of education and research, is being carried out to investigate the effects of EPO in severely burned patients. The primary endpoint is the time of complete healing of a split skin donor sample area. Furthermore, clinical parameters such as wound healing (Vancouver Scar Scale),

laboratory values, Quality of Life (SF-36), angiogenic effects, and gene- and protein expression patterns are to be determined. (EudraCT Number: 2006-002886-38, Protocol Number: 0506, ISRCT Number: ISRCTN95777824)

43.8 Conclusions and Future Perspectives on Skin Regenerative Therapies

It is obvious that regenerative therapies after skin loss have been executed especially with local topical approaches for a long time, without focusing on the underling biological processes taking place. Only recently, with a better molecular biological understanding of stem-cell and protein- based principles, we are able to customize regenerative therapeutic strategies which respect such fundamental biological principles. Perhaps the therapeutic use of EPO, which selectively triggers cell-protective and pro-regenerative effects, may play a key role in future developments of new therapeutics to enable and improve skin regeneration after partial skin loss.

Full skin loss will still remain a therapeutic challenge for clinicians. Since total skin loss necessitates skin transplantation or bioartificial generation of skin substitutes in such situations. Three key problems need to be solved in the future to optimize skin tissue engineering and tissue regeneration: creating a stable epidermo-dermal junction between the two major compartments dermis and epidermis, implementing a vascular supply in the dermal layer and supporting the construct with its functional cells and appendices (e.g. melanocytes, sweat and sebaceous glands, hair bulges etc.).

References

Aasen T, Belmonte JC (2010) Isolation and cultivation of human keratinocytes from skin or plucked hair for the generation of induced pluripotent stem cells. Nat Protoc 5(2):371–382. Epub 2010 Feb 4

Bader A, Machens HG (2010) Recombinant human EPO plays a pivotal role as a topical stem cell activator to reverse effects of damage to the skin in aging and trauma. Rejun Res 13:499

Bernaudin M et al (1999) A potential role for EPO in focal permanent cerebral ischemia in mice. J Cereb Blood Flow Metab 19:643–651

Bodó E et al (2007) Human hair follicles are an extrarenal source and a nonhematopoietic target of EPO. FASEB 21:3346–3354

Bolognia JL et al (2007) Dermatology. Mosby, St. Louis. ISBN 1-4160-2999-0

Brines M et al (2004) EPO mediated tissue protection through an EPO and common beta-subunit heteroreceptor. Proc Natl Acad Sci USA 101:14907–14912

Brines M et al (2008) EPO-mediated tissue protection: reducing collateral damage from primary injury response. J Int Med 264:405–432

Buemi M et al (2004) Recombinant human EPO stimulates angiogenesis and healing of ischemic skin wounds. Shock 22:169–173

Burns T et al (2006) Rook's textbook of dermatology CD-ROM. Wiley, Maiden. ISBN 1405131306

Converse JM et al (1975) Inosculation of vessels of skin graft and host bed: a fortuitous encounter. Br J Plast Surg 28:274–282
Egana JT et al (2009) The use of glandular-derived stem cells to improve vascularization in scaffold-mediated dermal regeneration. Biometerials 30:5918–5926
Ferguson MWJ et al (2004) Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. Phil Trans R Soc Lond B 359:839–850
Fernandes KJ et al (2008) Multipotent skin-derived precursors: adult neural crest-related precursors with therapeutic potential. Philos Trans R Soc Lond B Biol Sci 363:185–198
Ferri C et al (2007) Treatment of severe scleroderma skin ulcers with recombinant human EPO. Clin Exp Dermatol 32:287–290
Freedberg IM et al (2003) Fitzpatrick's dermatology in general medicine. McGraw-Hill, New York
Galeano M et al (2004) Recombinant human EPO stimulates angiogenesis and wound healing in the genetically diabetic mouse. Diabetes 53:2509–2517
Galeano M et al (2006) Recombinant human EPO improves angiogenesis and wound healing in experimental burn wounds. Crit Care Med 34:1139–1146
Hollander DA (2004) Reconstruction of extensive soft tissue loss by transplantation of dermal and epidermal equivalents. Zentralbl Chir 129:29–36
Keast DH, Fraser C (2004) Treatment of chronic skin ulcers in individuals with anaemia of chronic disease using rhEPO: a review of four cases. Osteotomy Wound Manage 50:64–70
Lorenz K et al (2008) Multilineage differentiation potential of human dermal skin-derived fibroblasts. Exp Dermatol 17:925–932
Lotze MT et al (2007) The great full death: damage-associated molecular pattern molecules and reduction/oxidation regulated immunity. Immunol Rev 220:60–81
McGrath JA et al (1971) Embryology of human skin. A review of ultrastructural studies. The Herman Beerman lecture. J Invest Dermatol 57:133–143
Meenakshi J et al (2005) Keloids and hyperthropic scars: a review. Indian J Plast Surg 38:175–179
Occleston NL et al (2008) Prevention and reduction of scarring in the skin by Transforming Growth Factor beta 3 (TGFbeta3): from laboratory discovery to clinical pharmaceutical. J Biomater Sci Polym Ed 19(8):1047–1063
Peterson TE et al (2007) EPO tecting the endothelium. Br J Pharmacol 150:823–825
Petschnik AE et al (2009) Phenotypic indications that human sweat glands are a rich source of nestin-positive stem cell populations. Br J Dermatol 162(2):380–383
Racila D, Bickenbach JR (2009) Are epidermal stem cells unique with respect to aging? Aging 1(8):746–750
Rapini RP (2005) Practical dermatopathology. Elsevier/Mosby, Philadelphia. ISBN 0-323-01198-5
Rezaeian F et al (2008) EPO protects critically perfused flap tissue. Ann Surg 248:919–929
Rezaeian F et al (2010) EPO-induced upregulation of endothelial nitric oxide synthase but not vascular endothelial growth factor prevents musculocutaneus tissue from ischemic damage. Lab Invest 90:40
Rockwell WB et al (1989) Keloids and hyperthropic scars: a comprehensive review. Plast Rec Surg 84:827–837
Rolletschek A, Wobus AM (2009) Induced human pluripotent stem cells: promises and open questions. Biol Chem 390:845–849
Schultz C et al (2008) Attenuation of monocyte proinflammatory cytokine responses to Neisseria meningitidis in children by erythropoietin. Clin Exp Immunol 154:187–191
Sorg H et al (2009) Effects of EPO in skin wound healing are dose related. FASEB 23:1–10
Takeuchi O et al (2007) Signalling pathways activated by microorganisms. Curr Opin Cell Biol 19:185–191
Viviani B et al (2005) EPO protects primary hipocamppal neurons increasing the expression of brain-derived neurotrophic factor. J Neurochem 93:412–421
Yazihan N et al (2008) Erythropoietin reduces lipopolysaccharide-induced cell damage and midkine secretion in U937 human histiocytic lymphoma cells. Adv Ther 25:502–514

Part V
Regulation and Ethics

Chapter 44
Regulatory Frameworks for Cell and Tissue Based Therapies in Europe and the USA

Gudrun Tiedemann and Sebastian C. Sethe

Abstract Whereas some basic therapies based on tissues and cells have been in clinical use for years, regulatory regimes applying to such applications have recently been revised and extended in Europe and in the US. Moreover, advances in regenerative medicine present new challenges and new types of products for regulation.

Both European and US regulators have developed rules to distinguish 'complex' cell therapies from their more established predecessors. In Europe, regulation of medicines and tissues and cells has now been supplemented by the regulation of 'Advanced Therapies' that is specifically relevant for regenerative medicine. We discuss the European legislative framework with reference to Germany and the UK as examples how the common rules are implemented. We also show how similar distinctions are made in the United States and consider the stance of the FDA on clinical development of novel cell therapies.

In conclusion, we briefly discuss whether the proposed regulatory regimes strike the appropriate balance between protecting patient safety and promoting innovation in regenerative medicine.

44.1 Introduction

Legal and regulatory provisions shape the medical innovation trajectory in major ways. To safeguard public and patient health, legislation is laid down to control the testing, manufacture, marketing and use of therapeutic products for human use.

G. Tiedemann, Ph.D. (✉)
Reference and Translation Center for Cardiac Stem Cell Therapy (RTC),
University of Rostock, Schillingallee 68, 18057 Rostock, Germany
e-mail: gudrun.tiedemann@med.uni-rostock.de

S.C. Sethe, Ph.D.
Lawford Davies Denoon, 14A Clerkenwell Green, London EC1R 0DP, UK

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_44,

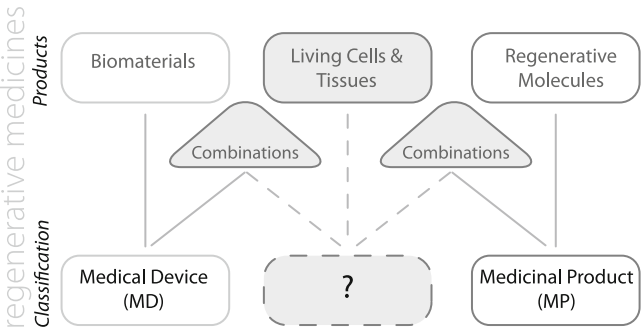


Fig. 44.1 Classification of regenerative products

Over the years, regulatory provisions have evolved to cover the medical sector more and more comprehensively and regulators are also struggling to keep up with novel scientific, technical and economic developments.

Advances in regenerative medicine result in a group of innovative and complex products that may involve living cells and tissues, regenerative molecules and biomaterials. These approaches to potential new treatments and long-term health protection stand for a step change in medicine.

A general distinction has traditionally been drawn between medical devices, pharmaceuticals and transplants. Some therapeutic approaches that could be classed as 'regenerative medicine' fit with existing regimes of regulatory oversight. For example, small molecules enhancing the regenerative capacity of endogenous stem cells would be classed as pharmaceuticals; a donated liver is a transplant. For others, the product classification may be ambiguous or confusing. For example, are genetically modified stem cells seeded on an implantable scaffold that contains a slow-release capsule which secretes chemical factors to promote angiogenesis a device (because of the scaffold), a drug (because of the factors), a transplant, a gene therapy or something else entirely? Moreover, can the new regenerative treatments be 'made to fit' existing categories or are there new and different considerations that innovators and regulators need to pursue?

Here, we will focus on the regulation of cell therapy and tissue engineered products (cell therapies in shorthand). Cell and tissue based therapies have long been left relatively unregulated, in part because these treatments were seen as more closely aligned to surgical interventions than the pharmaceutical market. Driven by scientific progress in regenerative medicine which has produced new and different types of the above 'borderline' complications, new legal provisions have been developed to regulate the cell therapies sector.

In this context, there has been considerable debate about what makes a regulatory regime in cell therapies regulation fit for purpose (see Fig. 44.1).

In this chapter, we will give a summary introduction to the regulatory regimes applicable to cell based therapies in Europe and the US and conclude with a brief discussion regarding the adequacy and effectiveness of these regulations.

44.2 Regulation of Cell and Tissue Based Therapies in Europe

In the European Union (EU) recent legislative efforts have specifically addressed cell and tissue engineering approaches. In order to understand how these initiatives take practical effect, a basic appreciation of European Law is required: A distinction can be made between European **Regulations** and European **Directives**. Whereas European Directives are considered to have direct *effect*, they first require implementation by national legislation in the individual Members State (MS). In contrast, European Regulations are *directly applicable* (yet may still be in need of substantiation in a national context). Therefore, although European Law may proscribe the regulatory parameters, the interpretation and implementation of these stipulations in individual MS may differ.

For this reason, after discussing the EU regulations in cell and tissue based therapies, we will look briefly at two MS – Germany and the United Kingdom (UK) as case studies for national implementation.

44.2.1 Basic Regulatory Domains

The three basic domains of medical products referenced in the introduction also exist in Europe:

Medical Devices
The core legal framework for medical devices consists of 3 directives **(the Device Directives)**: Directive 93/42/EEC covers medical devices generally. Directive 90/385/EEC concerns specifically active implantable medical devices. Many regenerative medicine approaches will fall under this scope. Also of interest is Directive 98/79/EC regarding in vitro diagnostic medical devices, such as tissue engineered toxicology assays. These directives have been supplemented over time by several modifying and implementing directives, including the last technical revision brought about by Directive 2007/47/EC.

A key regulatory component of bringing a medical device to the European market is the so called 'CE marking' to indicate conformity with the essential health and safety requirements. Depending on the class of product, conformity can be proven by the manufacturer or with the involvement of a **notified body**.[1]

Whether clinical trials are necessary to demonstrate safety and efficiency depends on the class of the product. Authorization for clinical trials is given by the competent authorities of the MS.

Pharmaceuticals
The nexus for regulation of small molecule drugs, complex biologics, and even herbal products, vitamins and minerals where used for medical treatment is

[1] A list of notified bodies can be found at http://ec.europa.eu/enterprise/newapproach/nando/

Directive 2001/83/EC (the Medicines Directive) which applies to medicinal products for human use intended to be placed on the market in Member States and either prepared industrially or manufactured by a method involving an industrial process. Under this legislation, all medicinal products in its scope require a Marketing Authorisation (MA) from the European Commission or the national competent authority of the MS to ensure quality, safety and efficacy before they can be sold commercially. Similar to the devices legislation, the Medicines Directive has also been extensively amended in order to incorporate new legislative agendas including, most recently, initiatives on regenerative medicine as will be discussed below.

Transplantation

Whole organ transplantation is not currently regulated at EU level, although efforts are underway to address this sector.[2] **Directive 2002/98/EC (the Blood Directive)** sets standards of quality and safety for the collection, testing, processing, storage and distribution of human blood and blood components. Although some blood products may be very relevant in regenerative medicine, we will not focus on this area here. **Directive 2004/23/EC (the Tissues and Cells Directive)** sets standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. This Directive is complemented by two technical directives (2006/17/EC and 2006/86/EC), which specify further detailed requirements. The Tissues and Cells Directive set standards that must be met when carrying out any activity involving tissues and cells intended for 'human application' (medical treatment of human patients). It could be thus be thought that the Tissues and Cells Directive is the relevant European regulatory instrument for cell therapies – however, the Directive only relates to cells which have been minimally manipulated such as in whole bone marrow transplantation and in fertility treatment. As we will see, most stem cell and tissue engineering therapies in regenerative medicine involve substantially manipulated cells or tissues and thus form part of a new regulatory paradigm on 'advanced therapies' which are regulated similar to pharmaceuticals under the Medicines Directive.

44.2.2 Legislation on Advanced Therapy Medicinal Products (ATMP)

After discussion and stakeholder consultation about regimes applicable to living cell based therapies, and in particular tissue engineered products, the European Commission established as *'lex specialis'* **Regulation (EC) No 1394/2007** on advanced therapy medicinal products (**the ATMP-Regulation**) as shown in Fig. 44.2.

From a legal implementation perspective, the ATMP Regulation has several elements: it amends other aspects of European medicines law most notably the

[2] Press release: MEMO/08/774, 08/12/2008.

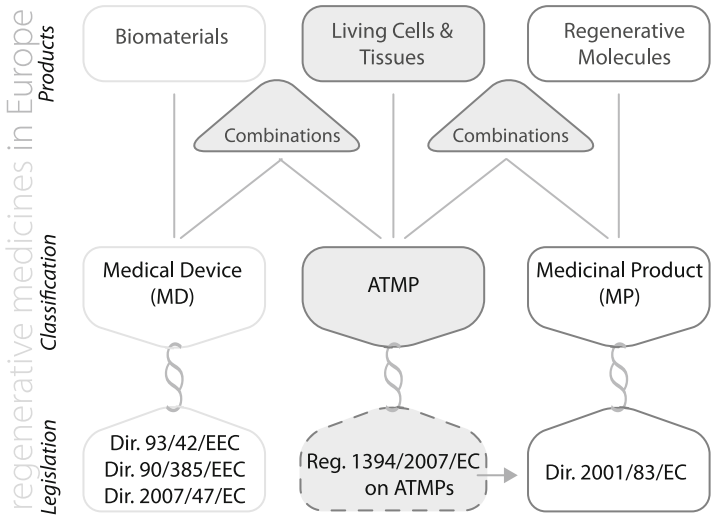


Fig. 44.2 Regulatory regimes for regenerative medicines in Europe

Medicines Directive; it contains some provisions which have direct applicability; and it contains some instructions for MS to establish further regulatory provisions and also tasks the European Commission and the European Medicines Agency with specific implementation steps. Figure 44.3 gives an overview of the follow-up amendments, legislation, guidelines and provisions engendered by the ATMP Regulation.

44.2.2.1 Types of Advanced Therapies

The ATMP Regulation establishes the concept of **Advanced Therapy Medicinal Products** (ATMP) – a category that is meant to encompass gene therapy, certain types of cell therapy and tissue engineering. With a circular cross-reference to Annex I Part IV of the Medicines Directive, (which has since been amended by Directive 2009/120/EC) the ATMP Regulation refers to products in these areas as 'gene therapy medicinal products' (GT), 'somatic cell therapy medicinal products' (SCT), and 'tissue engineered products' (TEP).

A comparison of the three ATMP-product-classes GT, SCT and TEP regarding definition, indication and active substance is shown in Fig. 44.4.

It should be pointed out that these definitions are regulatory constructs and not necessarily in line with scientific terminology (e.g. SCT may well include stem cell based treatments, even though the word 'somatic' is used).

The ATMP Regulation also recognises 'Combination Products' which are ATMP that incorporate, as an integral part of the product, one or more medical devices (Art.2 (d)).

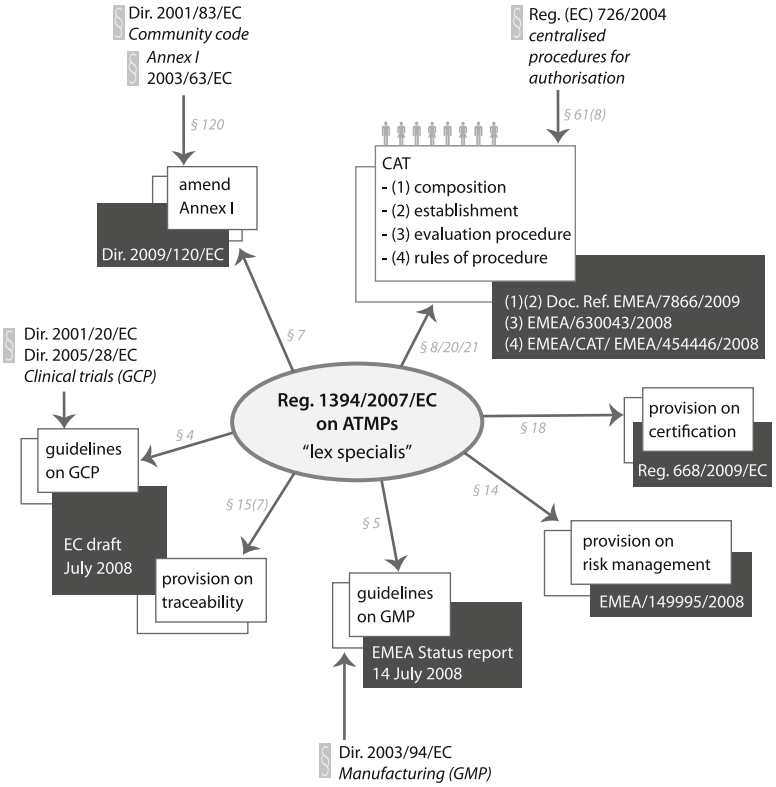


Fig. 44.3 Integration of Regulation (EC) 1397/2007 into the European regulatory framework: Implementation plan (black font on white background) and the current implementation status (white font on dark background)

The ATMP Regulation has no impact on national legislation prohibiting or restricting the use of certain type of human or animal cells (for example embryonic stem cells) and aims not to interfere with MS policy on whether to allow the use of any specific type of human cells. Products modifying the germ line genetic identity of human beings and products derived from human-animal hybrids or chimeras are excluded from the ATMP Regulation, but Xenotransplantation is specifically included.

As one can see from the definitions listed in Fig. 44.4 a lot turns on a decision of whether cells/tissues are 'substantially manipulated'. TEP make a similar reference to cells/tissues which are 'engineered'.

The ATMP Regulations specify that manipulations which shall **not** be considered as 'substantial manipulations' include: cutting, grinding, shaping, centrifugation, soaking in antibiotic or antimicrobial solutions, sterilization, irradiation, cell separation, concentration or purification, filtering, lyophilisation, freezing, cryopreservation and vitrification.

	gene therapy medicinal product GT	somatic cell therapy medicinal product SCT	tissue engineered product TEP
full definition	Directive 2001/83/EC Annex I Part IV –2.1	Directive 2001/83/EC Annex I Part IV –2.2	Reg. (EC) 1394/2007/EC Art.2(b)
indication	administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence;	treating, preventing or diagnosing a disease through pharmacological, immunological or metabolic action	regenerating, repairing or replacing a human tissue
active substance	recombinant nucleic acid	(engineered) cells or tissues that have been subject to substantial manipulation so that biological characteristics, physiological functions or structural properties relevant for the intended clinical use have been altered, or that are not intended to be used for the same essential function(s) in the recipient and the donor	
exclusions	vaccines against infectious diseases		products containing or consisting exclusively of non-viable human or animal cells tissues, which do not contain any viable cells or tissues and which do not act principally by pharmacological, immunological or metabolic action

Fig. 44.4 Definitions of ATMPs: GT-, SCT-, and TE-products

A similar classification problem can exist where tissues and cells are not intended to be used for the same essential function (so called 'non homologous use').

In summary, tissues and cells are 'elevated' to ATMP when they EITHER are 'substantially manipulated' OR 'for non-homologous use (see Fig. 44.5) – or both.

In effect, this means that the great majority of regenerative medicine therapies will be covered by the ATMP Regulation. Nonetheless, this determination must be made for each product individually. The Commission anticipated that such classification questions may lead to problems initially and CAT have established a free classification procedure which is supposed to feed back a classification recommendation to the questioner within 60 days. The results of these determinations are published to provide other innovators with a list of examples.

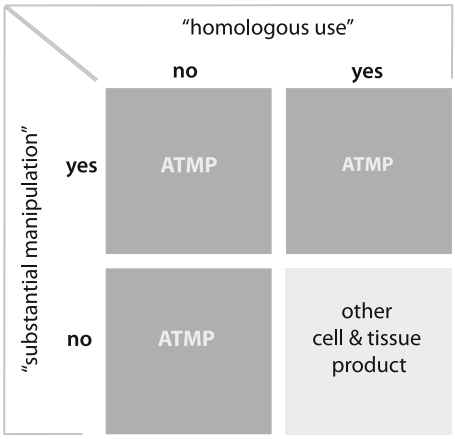


Fig. 44.5 Cells or tissue products in and out of the definition of an ATMP

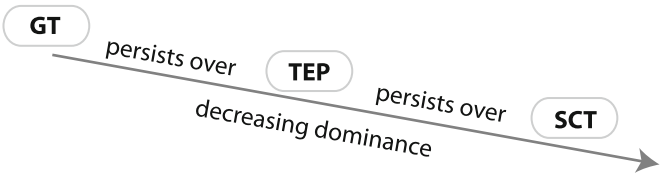


Fig. 44.6 'Dominance' of ATMP classification

44.2.2.2 Dual Classification

The ATMP-Regulation provides some general rules on classification of an ATMP that fulfils multiple characteristics:

Where a product contains viable cells or tissues, the pharmacological, immunological or metabolic action of those cells or tissues shall be considered as the principal mode of action of the product. A product with mixed characteristics is classified only by according to the dominant characteristic in the following order (Fig. 44.6):

Whereas it is important to point out the primacy of GT in this arrangement, we focus here on TEP and SCT products. In both cases, it may sometimes be difficult to determine whether a product qualifies as covered by the ATMP Regulation or whether it is covered 'only' by the Tissues and Cells Directive.

44.2.2.3 'Exemption §28(2)' from the Scope

Because the ATMP regulations builds on the Medicine Directive, its scope is limited to products which are intended to be placed on the market in MS and which are

either prepared industrially or manufactured by a method involving an industrial process. If an ATMP is **not** prepared industrially or manufactured by a method involving an industrial process, **and not** intended to be placed on the market in the Member State it is out of the scope of the ATMP-Regulation.

In order to avoid these cell-and tissue-products to be completely exempted from pharmaceutical legislation, the ATMP-Regulation (Art. 28(2)) amends Art.3 of Directive 2001/83/EC with the so-called 'Hospital Exemption' related to ATMPs which are prepared on a non-routine basis according to specific quality standards, and used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner, in order to comply with an individual medical prescription for a custom-made product for an individual patient (see Fig. 44.7).

Member States are requested to lay down rules for authorising these products by the national Competent Authority whilst at the same time ensuring that relevant Community rules related to quality and safety are not undermined.

While searching for 'exemptions' to the process of marketing authorisation, another, similar provision may be of interest that predates the ATMP Regulation and applies equally to all other medicines: According to Art.5.1 of the Medicines Directive, a MS may, in order to fulfil special needs, exclude a medicinal product from the provisions of the Medicines Directive altogether if that product is supplied in response to a bona fide unsolicited order, formulated in accordance with the specifications of an authorised health-care professional for use by an individual patient under his direct personal responsibility (see Fig. 44.8).

Whether this provision is useful and applicable will depend not only on the circumstances of the individual case but also on the extent that the individual MS has recognised and interpreted the provision.

44.2.3 Interactions with Regulatory Bodies

44.2.3.1 Marketing Authorisation

In order to place an ATMP product on the market in the EU, the manufacturer needs to obtain marketing authorisation (MA) from the European Commission.

All ATMP are subject to a centralised MA procedure which involves a single scientific evaluation of the quality, safety and efficacy of the product which is carried out by the European Medicines Agency (EMEA)[3] as established by Regulation (EC) No 726/2004.

For ATMPs which were 'legally on the market' in accordance with national or Community legislation on 30 December 2008 a transitional period of 3 years for SCT and GT (30 December 2011) and 4 years for TEPs (30 December 2012) is granted.

[3] Following a recent rebranding, the European Medicines Agency is no longer using the acronym EMEA, but is also not using EMA. Here we have opted for EMEA to avoid confusion for those used to the old abbreviation.

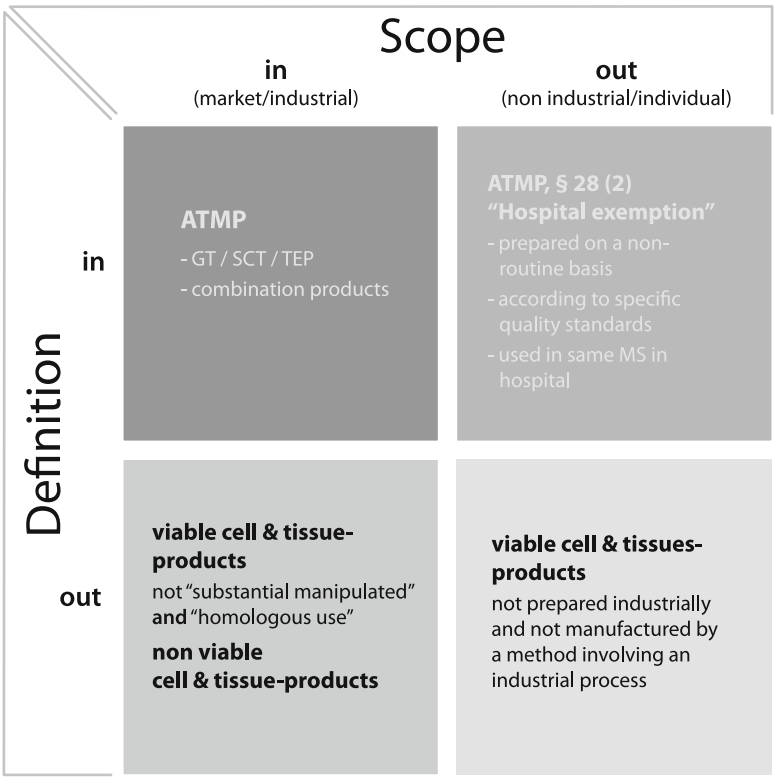


Fig. 44.7 Cells or tissue products in and out of the definition of an ATMP

	2001/83/EC	2001/83/EC Art. 3(7)	2001/83/EC Art.5.1.
Authorised by	European Commission	treating medical practitioner	treating health care professional
Conditions	marketing /manufacturing authorisation	-individual medical prescription -custom-made product -individual patient	-bona fide unsolicited order -individual patient
Requirements	safety, efficacy etc	non-routine basis	special needs
Location of treatment	/	a hospital	/
Location of manufacture	any accredited facility anywhere	prepared and used in the same MS	manufactured in an eligible MS or imported to an eligible MS

Fig. 44.8 Some of the main differences in scope between ATMP produced under the standard provisions, Art. Directive 2001/83/EC Art. 3(7), and Art.5.1

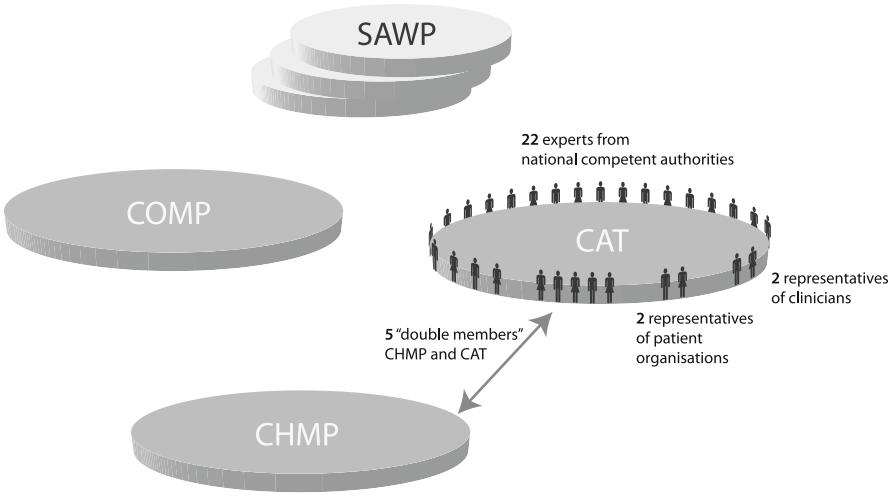


Fig. 44.9 Committees involved in evaluation of ATMPs

MA is not required where the product is still undergoing development in clinical trials.

44.2.3.2 Committee for Advanced Therapies (CAT)

The evaluation of ATMPs often requires very specific expertise. For this reason a new and multidisciplinary expert committee 'Committee for Advanced Therapies' (CAT) within EMEA has been established, to assess ATMPs and to follow scientific developments in the field (see Fig. 44.9). The names and scientific qualifications of the members are made public by the Agency. The CAT is responsible for preparing a draft opinion on the quality, safety and efficacy of each ATMP – including combined ATMPs – for final opinion by the Committee for Medicinal Products for Human Use (CHMP). The CHMP delivers this opinion to the Commission for final approval.

For scientific consistency and the efficiency of the system, the coordination between the CAT and the other Committees, advisory groups and working parties, notably the CHMP, the Committee on Orphan Medicinal Products (COMP), and the Scientific Advice Working Party (SAWP) must be ensured.

44.2.3.3 The Role of National Regulators

Whereas ATMP have to pursue the centralised European route, for other regenerative medicine products it may be possible to gain national approval in individual MS and subsequently European-wide approval under the mutual recognition procedures. EMEA has no scientific assessors on its own and relies on outsourcing its licensing

activities to national authorities. In the young field of regenerative medicine, arguably the most important role for national regulatory authorities however, is in regulating the conduct of clinical trials. Clinical trial authorisation – as well as manufacturing authorization of the clinical trial samples – is required in each MS where a trial is being undertaken. Some MS further differentiate between national and regional authorities.

44.2.3.4 Fee Reductions

Specific incentives for small and medium sized enterprises (SMEs) exist in the ATMP area. Additional procedures are offered to support applicants in the development process.

Any applicant or holder of a marketing authorisation may request advice from the Agency on the design and conduct of pharmacovigilance and of the risk management system. There are specific incentives of 90% fee reduction for SMEs and 65% for others. If an applicant is SME or a hospital and can prove there is a particular public health interest in the Community he can get additional fee reductions: 50% fee reduction on MA fee and 50% post authorisation activities for 1 year.

44.2.3.5 Certification of Quality and Non-clinical Data

A new certification system aims at giving SMEs an incentive to develop ATMPs. Under this scheme, the Regulator can 'certify' data as being of sufficiently high quality for regulatory consideration. It is expected that innovators will then be able to raise capital for further R&D. The scope of the evaluation is to certify that each submitted study complies with the relevant scientific and technical requirement set out in the Annex I to Directive 2001/83/EC and adequately follows state-of-the-art scientific standards and guidelines.

SMEs may submit to the Agency all relevant quality and, where available, non-clinical data required in accordance with modules 3 and 4 of Annex I to Directive 2001/83/EC, for scientific evaluation and certification.

The Commission has laid down provisions for the evaluation and certification of the data.

Not a marketing authorisation: The certification procedure is independent from a future application for MA. But it could facilitate the evaluation of any future application for clinical trial authorisation or a marketing authorisation application (MAA), provided that these applications are based on the same data.

Not 'legally binding' for the Agency: A certificate is not binding with regard to any future regulatory procedure and all relevant data should be submitted again for the purpose of any future regulatory procedure.

Mostly quality and, where available, non clinical data: The certification procedure covers only a scientific evaluation of experimental data (quality/non-clinical) already generated. Advice for further development will have to be obtained by the Scientific Advice procedure.

The certificate cannot conclude on the adequacy of the studies submitted to be further developed in a clinical trial. This is under the responsibility of the National Competent Authorities where the clinical trial will be conducted.

Whether such a certification scheme will prove a worthwhile investment for innovators remains to be seen. Until 2012 there was only one certification procedure conducted by the CAT. The reason may be that the procedure is not open for Academia where about 60% of the early development is done. This has to be changed in future.

44.2.3.6 Specific Requirements

Part IV, Annex I of the Directive 2001/83/EC lays down detailed scientific and technical requirements regarding the testing of medicinal products for human use and describes the format requirements (Modules 1–5) for MA. This section was recently amended by Directive 2009/120/EC specifically to address ATMP. Member States shall bring into force the laws, regulations and administrative provisions necessary to comply with Directive 2009/120/EC by 5 April 2010 at the latest.

The regulations specify a number of requirements. A few examples:

Cell sources: Information on donation, procurement and testing shall be provided. Animal cells or tissues are expressly not excluded but specific acceptance criteria must be provided. If 'non-healthy' cells or tissues are used as starting materials, their use shall be justified. Problematically, if allogeneic cell populations are being pooled, the pooling strategies and measures to ensure traceability shall be described. It is still unclear what constraints this imposes on 'rollover' cell pools.

Pre-clinical development: The Regulations suggest that 'The use of homologous models (e.g. mouse cells analysed in mice) or disease mimicking models shall be considered, especially for immunogenicity and immunotoxicity studies'. Different scientific opinions exist on the value of such studies. The regulations state that 'conventional pharmacokinetic studies to investigate absorption, distribution, metabolism and excretion shall not be required'. However, parameters such as viability, longevity, distribution, growth, differentiation and migration of cells shall be investigated, unless otherwise duly justified. Given the reported difficulties in cell tracking, this requirement alone may provide a significant barrier to development.

Risk analysis: Risk factors that may be considered include: the origin of the cells (autologous, allogeneic, xenogeneic), the ability to proliferate and/or differentiate and to initiate an immune response, the level of cell manipulation, the combination of cells with bioactive molecules or structural materials, the long time functionality, the risk of oncogenicity and the mode of administration or use.

The manufacturing process involves the emulation of the concept of a 'production batch' used in the context of mainstream pharmaceuticals. Manufacturing must be validated to ensure "batch consistency" and "the proper differentiation state and the cell function with additional substances throughout the manufacture" – this would

seem to place significant technical requirements on manufacturers in handling an inherently heterogeneous product. The regulations suggests that normally, the functional integrity of the cells should be tested at the moment of application/administration, but specify that if certain release tests cannot be performed on the active substance or finished product, but only on key intermediates and/or as in-process testing, this needs to be justified.

Risk-based Approach: in January 2012 the CAT came over with a new draft guideline on the risk-based approach according to Annex I, part IV of Directive 2001/83/EC applied to ATMP. It is a strategy aiming to determine the extent of quality, non-clinical and clinical data to be included in the Marketing Authorization Application (MAA), in accordance with the scientific guidelines relating to the quality, safety and efficacy of medicinal products and to justify any deviation from the technical requirements. It is not the intention to provide a rigid classification system of different risks but rather to exemplify the concept by using several examples with different risk profiles. This may be a worthwhile instrument leading through the complex development process in a fruitful dialog with the authorities.

44.2.3.7 Specific Guidelines on Good Clinical Practice (GCP) and Traceability

Clinical trials on ATMPs have to be conducted in accordance with the overarching principles and the ethical requirements laid down in Directive 2001/20/EC for good clinical practice. However, Commission Directive 2005/28/EC laying down principles and detailed guidelines for good clinical practice, as well as the requirements for authorisation of the manufacturing have to be adapted to ATMPs. Draft Guidance by the Commission thus far simply references 2005/28/EC and CPMP/ICH/135/95 without adding many further specifications regarding, inter alia, the investigators brochure, the clinical protocol, ethics quality control etc. However, this approach brings a particular emphasis to the requirements for traceability that the document selectively focuses on.

The system has to ensure coherence and compatibility with traceability requirements in the Tissue and Cells Directive. Notably, the traceability system must also respect the provisions laid down in Directive 95/46/EC on data protection, which are considered to be particularly stringent in international comparison. For example, because the European Commission does not regard the privacy laws in the US as adequate, the transfer of patient data, to the USA is prohibited except under special 'safe harbour' agreements.

44.2.3.8 Guideline on Safety and Efficacy Follow Up – Risk Management of ATMPs

In addition to the requirements for pharmacovigilance laid down in Articles 21 to 29 of Regulation (EC) No 726/2004, the MA-application for an ATMP shall lay

down measures envisaged to ensure the follow-up of efficacy of ATMPs and of adverse reactions thereto. The Commission requires a risk management system designed to identify, characterise, prevent or minimise risks related to AMPs, including an evaluation of the effectiveness of that system, be set up. EMEA may stipulate that specific post marketing studies be carried out.

If serious adverse events or reactions occur in relation to a combined ATMP, there is an obligation for EMEA to inform relevant national competent authorities.

44.3 Examples: Germany and United Kingdom

As explained above, although European Regulations on regenerative medicine impose an ever greater degree of uniformity on regulatory standards across Europe, Members States retain some leeway in implementing those provisions into national law. We have also seen that the ATMP 'Hospitals Exemption' is expressly delegated to National Competent Authorities. Here, we will briefly provide two illustrations on how European regulations are incorporated into the national framework by brief reference to Germany and the UK.

44.3.1 Germany

There are two main Competent Authorities in Germany: the *Bundesinstitut für Arzneimittel und Medizinprodukte (*BfArM – Federal Institute for Drugs and Medical Devices) and the *Paul-Ehrlich-Institut* (PEI – Federal Institute for Vaccines und Biomedical Drugs). The latter is responsible for cell and tissue products. In addition to these federal authorities there are cooperating local authorities, which have specific functions. The German approval and authorisation requirements are laid down in the German MP-Act the *'Arzneimittelgesetz'* (AMG).

For living cell and tissue based medicines the relationship between Community and German legislation is shown in Fig. 44.10.

44.3.1.1 Basic Tissue and Cell Treatments in Germany

Since 2007 the Tissues and Cells Directive has been transposed to the German law, as amendments to the transfusion-, transplantation- and pharmaceutical regulation. The definitions and specific rules for classic tissue-preparations (*'Gewebezubereitungen')* have been laid down in the 14th Amendment ('14te Novelle') of the AMG.

'Classic' tissue/tissue-preparations are defined as MP in §4(30) AMG, regarding the German Transplantation Act §1a Nr. 4 (TPG). If these products are **produced industrially** the requirements are – as for any other MP – the manufacturing

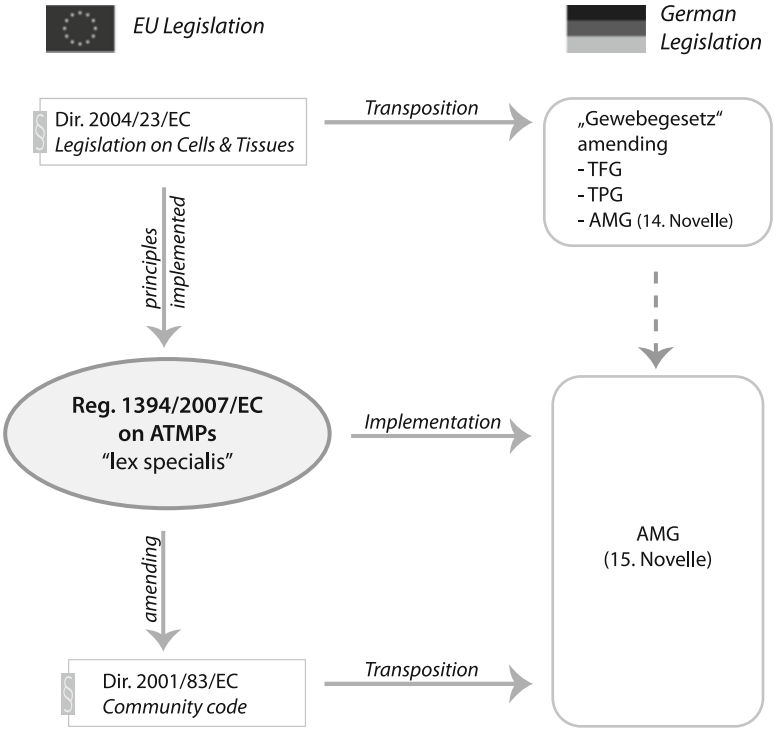


Fig. 44.10 Relationship between Community and German legislation

authorisation regarding §13(1) AMG by the local authority (after consultation with the PEI) and MA through §21(1) AMG, including clinical trials.

If the tissue preparations however are **not processed with industrial methods and where the methods are well-known in the Community**, specific national authorisation provisions for manufacturing as well as for use are laid down in the AMG.

For **manufacturing** again the respective local authority is responsible but the manufacturer has to fulfil only reduced requirements for processing and testing (§20c AMG) – in addition to the general requirements for donation, procurement and testing laid down in §20b AMG (which is also applicable for the procurement of autologous blood, often required for the preparation of TEPs).

The **authorisation for use** is the so called *'Genehmigung'* (§21a AMG), issued by the PEI. The requirements are – compared to the 'normal' dossier for MA – rather similar but reduced, particularly regarding clinical trials.

According to §20d AMG tissues/tissue preparations are **excluded** from these provisions, if they are not placed on the market and are processed and applied under the responsibility of a physician (*'Einhandprinzip'*). Tissues, which are procured and re-applied within the medical operation without any change to their material composition, are entirely excluded from the scope of the AMG (§4a (3)).

44.3.1.2 ATMP in Germany

The implementation of the ATMP Regulation with its follow up effects has led to new further amendments to the AMG. Since July 2009 ATMPs are implemented in the '15th Novelle' under the term '*Arzneimittel für neuartige Therapien*' (§4(9) AMG). GMP-manufacturing (§13(1) AMG) and centralised MA (§21 AMG) are required.

For the exemption according to Article 28(2) of the ATMP-Regulation for 'non-routine' ATMPs §4b AMG lays down *'Sondervorschriften für Arzneimittel für neuartige Therapien*'. In the following we will focus on the German provisions for this 'Hospitals Exemption'.

44.3.1.3 'Hospital Exemption' in Germany

The specific 'non routine' provisions are laid down in §4b (1 and 2) of the AMG and apply to ATMPs, which are:

- prescribed by a physician for an individual patient as a custom-made preparation,
- applied under the responsibility of a physician in a specialised health care unit
- ***manufactured on a non-routine basis*** according to specific quality standards.

  Here *'manufactured on a non-routine basis'* means in particular ATMPs

  - which are manufactured on a small scale and where – on the basis of routine production – the product has to be ***individually modified because of a medical indication for a single patient***, or
  - products not yet manufactured in a sufficient number to lay down the necessary results for a comprehensive evaluation.

  The German provisions for these 'non-routine' ATMPs are in detail:

- **no** need for MA according (§21 AMG) (as there is **no** placing on the market (§43 AMG))
- traceability and pharmacovigilance administered via competent local authority and/or PEI (but equivalent to the rules on Community level)
- quality standards for production by manufacturing authorisation (*'Herstellerlaubnis')* regarding §13 AMG via local authority and PEI (same authorisation as for 'routine ATMPs')
- if the ATMPs are "handed over to others" authorisation through '*Genehmigung*' (§21a(2–8)AMG) via PEI (specific quality standards: template for authorisation corresponds in general with the Common Technical Document (CTD) for approval but the 5 CTD-modules for quality, preclinical and clinical data, summaries and registration are abbreviated versions) is required.

If there is doubt whether an ATMP falls under the provision *'Genehmigung'* or not, the relevant local competent authority is responsible to decide this on request of the applicant and after consultation with the PEI.

The authorisation will be withdrawn, when the prerequisites for the 'exemption' are not or no more fulfilled. At defined time points the owner of the authorisation has to report to the PEI about the scale of production and/or the consolidated findings for the evaluation of the MP.

44.3.2 United Kingdom

44.3.2.1 Basic Tissue and Cell Treatments in the UK

The regulation on tissues and cells which are not classified ATMP is largely covered by the Human Tissue Act 2004 (HTA). Following a national scandal into unauthorised retention of organs for research, the Human Tissue Act is unusual in a European context in covering the storage of human tissue not just for purposes of the Tissue and Cells Directive but also for clinical and other research.

The UK Human Tissue Authority has issued Directions under Art.26(7) of the Human Tissue Act to address the European requirements: HTA Directions 001/2006 implement the requirements of the Tissue and Cells Directive and technical Directive 2006/17/EC including standards relating to procurement, distribution, donor selection and evaluation, and the transport of tissues and cells. HTA Directions 002/2007 implement technical Directive 2006/86/EC on facilities and equipment, quality management and review, confidentiality, processing and storage and the reporting of serious adverse events and reactions. HTA Directions 004/2007 regulate to the import of tissues and cells from outside the EU.

44.3.2.2 ATMP in the UK

Cell therapies which are classed as ATMP on the other hand are primarily regulated as normal medicines under the Medicines Act 1968 which – with its vast number of amendments – "has become a very complex and fragmented set of legal provisions" the structure of which is currently under review.[4] Clinical trials for ATMP will be regulated mainly under the Medicine for Human Use (Clinical Trials) Regulations 2004 by the UK Medicines and Healthcare products Regulatory Agency (MHRA).

44.3.2.3 Hospitals Exemption in the UK

MHRA has consulted in this context not only on the implementation of the 'Hospitals Exemption' (Art.3 (7) 2001/83/EC as amended) but also on the re-framing of the UK 'Specials' regime (ie the national arrangements set up under a derogation in Article 5.1 of Directive 2001/83/EC).

[4] MHRA Concept paper on the project to consolidate and review medicines legislation; Jan 2009.

Where a number of different products are under consideration the question of whether preparation is non routine will be considered separately in relation to each product prepared by that operator.

MHRA will take into account the overall numbers of the product prepared by the operator, the regularity/frequency of production, and the time period over which the preparation of that product has become established. The Agency would not, for example, accept an argument that depended on the premise that all autologous ATMPs were by definition different products, where their intended use, manufacturing processes and final product presentation are the same.

MHRA suggests that it should typically be possible to determine within a period of 1–3 years where the scale and frequency of production means that preparation has become routine, but where some months are elapsing between each preparation, a significantly longer period may need to elapse before the preparation could be reasonably regarded as routine.

A manufacturer needs to obtain a hospitals exemption manufacturer's licence from the MHRA. The licence will authorise the manufacture of particular categories of ATMPs (gene therapy, somatic cell therapy or tissue engineered product) rather than individual products. ATMPs made and used under the exemption must comply with the principles of GMP as stipulated by the European Commission. The MHRA will inspect for compliance with GMP and review an annual return on this activity.

44.4 Regulation of Cell and Tissue Based Therapies in the USA

44.4.1 Legislative Framework

We have seen that for Europe, an understanding of the relationship between Community and Member States legislation is useful for a perspective on the regulation of cell therapies. Similarly the US constitution provides a basis for medicines regulation in the USA. Congress regulates interstate commerce, and in this context the authority to regulate drugs, devices, and biological products was delegated to the Food and Drug Administration (FDA) by the federal Food, Drug and Cosmetics Act.

As shown in Fig. 44.11 the FDA divides regulatory oversight in this area among the Center for Drugs, Evaluation and Research (CDER) which deals with 'chemical' pharmaceuticals, the Center for Biologics, Evaluation and Research (CBER) which deals with 'complex' biological treatments and the Center for Devices and Radiological Health (CDRH) that deals with medical devices.

CBER will likely be the most important centre for innovators in this area and within CBER the Office of Cellular, Tissue and Gene Therapies (OCTGT) is comprised of three Divisions:

- Division of Cellular and Gene Therapies (DCGT)
- Division of Clinical Evaluation and Pharmacology/Toxicology (DCEPT)
- Division of Human Tissues (DHT)

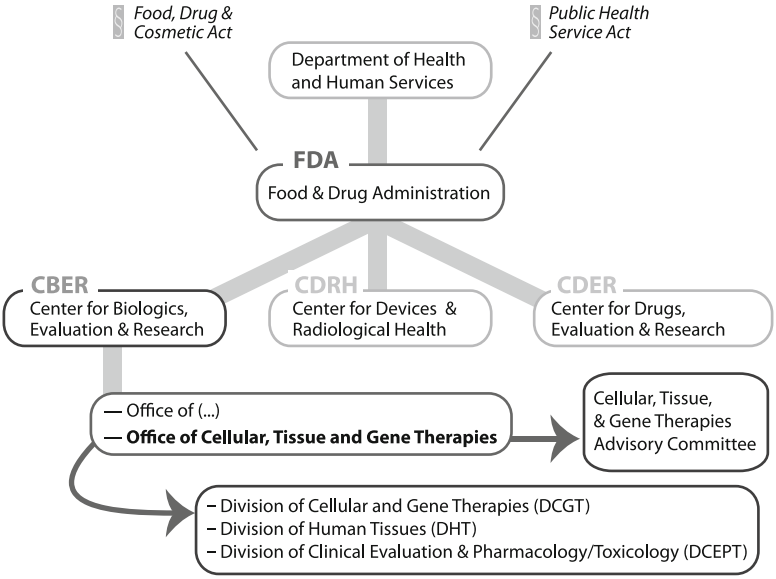


Fig. 44.11 Organigram of FDA with focus on cellular therapies (Note that this is a snapshot, other FDA institutions may be of relevance, e.g. within CBER the Office of Compliance and Biologics Quality, or the Office of Communication, Outreach and Development)

Contact details of relevant personnel can be accessed at the FDA website.[5]

Whereas FDA is the ultimate respondent on regulatory affairs, also of interest in this sector is the important role of voluntary accreditation and certification programs such as by the American Association of Tissue Banks (AATB) and Foundation for the Accreditation of Cellular Therapy (FACT).

FDA considers "articles containing or consisting of human cells or tissues that are intended for implantation, transplantation, infusion, or transfer into a human recipient" as **human cellular-and tissue-based products (HCT/Ps)**. However, the designation as HCT/P in itself does not determine how the product will be considered by the FDA. In the past, cell and tissue therapies have been exempt from product regulation because of their stronger association to medical practice than to industrial manufacture. During the last decade however, steps were taken to bring the sector under stronger regulatory supervision.[6]

In the regenerative medicine area, the first determination will be whether the product requires marketing authorisation. The product's classification determines the regulatory scrutiny of clinical R&D and marketing authorisation. It also determines the FDA branch with lead responsibility for the product.

[5] Currently at http://www.fda.gov/AboutFDA/CentersOffices/CBER/ucm123224.htm

[6] Beginning with the "Reinventing the Regulation of Human Tissue" discussion paper CBER February 1997.

44.4.1.1 Unaltered or Manipulated

Organs, blood products and tissues do not require marketing authorisation, but they still require compliance with regulatory standards.

CBER does not regulate the transplantation of **vascularised human organ transplants** and blood vessels recovered with an organ. These are overseen by the Health Resources Services Administration (HRSA) (although the position in the case of a vascularised tissue engineered human organ may 1 day be of interest).

Blood and Blood Products are sui generis products covered under CP 7342.001 "Inspection of Licensed and Unlicensed Blood Banks, Brokers, Reference Laboratories, and Contractors"; and CP 7342.002 "Inspection of Source Plasma Establishments".

Also excluded are secreted or extracted human products, such as milk, collagen, and cell factors; (semen *is* considered an HCT/P); Cells, tissues, and organs derived from animals other than humans; and in vitro diagnostic products.

Tissues: Some HCT/Ps are regulated solely under section 361of the US Public Health Service (PHS) Act and the regulations in 21 CFR Part 1271 (see Fig. 44.12).

Tissues and cells under this category include bone (including demineralized bone), ligaments, tendons, fascia, cartilage, ocular tissue (corneas and sclera), skin, arteries and veins (except umbilical cord veins), pericardium, amniotic membrane (when used alone, without added cells for ocular repair), dura mater, heart valve allografts, semen, oocytes and embryos (but not embryonic stem cells). The category also includes "hematopoietic stem/progenitor cells derived from peripheral and cord blood" – this is likely a significant borderline area in the context of regenerative medicine, not least because with these cells there is no consensus on what entails an original and relevant characteristic. However, the Administration advises that 'propagation' and 'pharmacological treatment' are at any rate 'kick-up factors' that constitute 'substantial manipulation' and bring blood stem cells into the area of products requiring marketing authorisation.

Where doubt exists, the FDA Tissue Reference Group (TRG) aims to provide a single reference point for HCT/Ps classification questions – however, an alternative and ultimately more authoritative route exists through the Office of Combination Products (OCP). The TRG is composed of representatives from CBER and CDRH and attended by a liaison from OCP. The group will issue guidance to applicants within 60 days on whether the product is regulated solely as a tissue. Similarly, a request for designation to OCP will yield a response within 60 days, with an opportunity to request reconsideration after 15 days of receiving the opinion, to which OCP must respond within 15 days.

44.4.1.2 Drug, Biologic or Device

If the HTC/P is considered substantially manipulated, so as to be regulated as a medicinal product the question arises what type of product it would then be. FDA seeks to determine this by focusing on the 'primary mode of action' of the therapy.

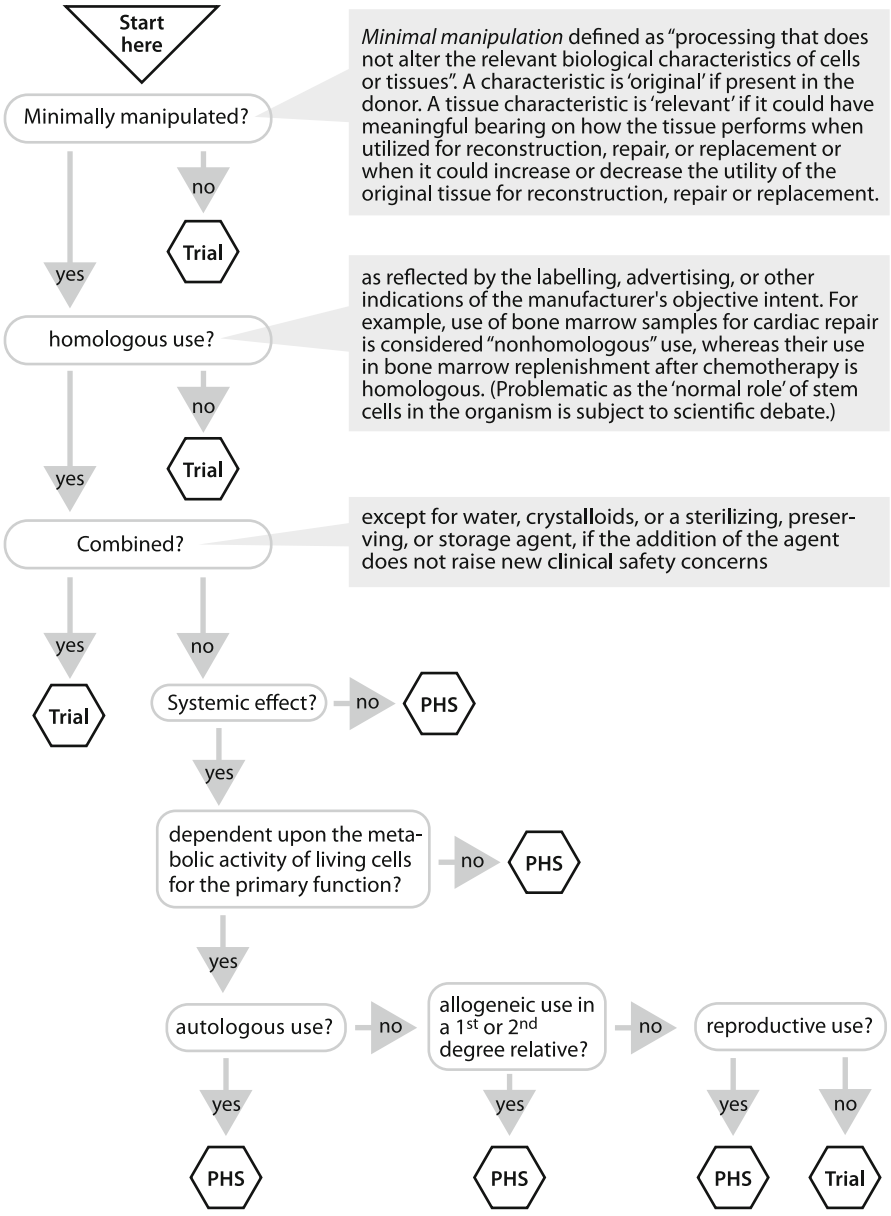


Fig. 44.12 Decision tree relating to whether a human tissue or cell product is regulated exclusively under Sec.361 of the US Public Health Service Act (PHS) or requires a license, approval, or clearance as part of a premarket review (Trial)

If the 'primary mode of action' is that of a drug, the product is assigned to CDER, if that of a device to CDRH, and biologics to CBER.

Drug is an article intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in humans or animals, and an article (other than food) intended to affect the structure or any function of the body (42 USC 262(a)).

This category is oriented towards 'established', 'pill-type' products. Thus, very few products in regenerative medicine will be considered as drugs.

Device means an instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent, or other similar or related article, including any component, part, or accessory, which is intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, (…) or intended to affect the structure or any function of the body (…) and which does not achieve any of its principal intended purposes through chemical action within or on the body and which is not dependent upon being metabolized for the achievement of any of its principal intended purposes (21 USC 201(h)). The latter provisions seem to preclude certain bioresorbable scaffolds.

Biologic is defined as a virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product or analogous product, (…) applicable to the prevention, treatment, or cure of diseases or injuries of man (42 USC 262(a)). Most HCT/Ps that have been substantially altered will fall into this category.

There is no statutory definition of what constitutes primary mode of action. FDA has issued guidance[7] where the mode of action is defined as the means by which a product achieves its intended therapeutic effect, and –for combination products– the single mode that is expected to make the greatest contribution to the overall intended therapeutic effects. Sponsors can instigate a 'Request for Designation' (21CFR §3.7 – all §§ in this section are under 21 CFR) but in the past the classification of a product as either a device or biologic has sometimes appeared arbitrary. To complicate matters CDER now review certain biologics including: monoclonal antibodies for in vivo use; cytokines, growth factors, enzymes, immunomodulators, and thrombolytics; proteins intended for therapeutic use that are extracted from animals or microorganisms, including recombinant versions of these products (except clotting factors); and other non-invasive immunotherapies.

OCP publishes jurisdictional updates of decisions rendered on sample products.

44.4.1.3 Regulation of 'Unaltered' Tissue

Although, as we have seen, the issues of classification are not always clear-cut, it is unlikely that many advanced tissue or cell based products will be treated as tissue in this category. However, the regulations relating to tissues are still of prime relevance

[7] Definition of primary mode of action of a combination product. Fed Regist 70(164, Aug 25):49848–49862.

to innovators who –perhaps in a trans-Atlantic collaboration– use tissues and cells as 'raw material' for further or future development (e.g. blood stem cells as a component, or embryos for the derivation of embryonic stem cell lines). Similar to the European situation, the regulatory provisions that apply to the procurement of tissue will also be relevant for the further development of cell therapy and tissue engineered products. For example, the donor testing and eligibility criteria will apply to both contexts.

In the past, innovators have sometimes been able to convince regulators of the acceptability of a tissue component (e.g. a cell line) 'post hoc' with safety data, but the preferred approach will be one of integrating regulatory standards throughout the product development chain. We will therefore quickly reference some of the relevant provisions in this section.

Domestic or foreign establishments that manufacture or import HCT/P into the US must register with FDA and submit a list of each a HCT/P manufactured. CBER maintains a listing of registered HCT/P establishments on which over 100 foreign stem cell procurement facilities are listed.[8] Satellite recovery establishment only provide temporary storage of recovered HCT/Ps and may perform no other activity or manufacturing step.

HCT/P establishments must screen and test HCT/P donors for risk factors for, and clinical evidence of, relevant communicable disease agents and diseases and communicable disease risks associated with xenotransplantation. These procedures must be designed to ensure compliance with the requirements of subpart 21 CFR 1271 C. Donor eligibility determination must be based upon the results of donor screening (§ 1271.75) and donor testing (§§ 1271.80 and 1271.85). Certain records must accompany the HCT/P at all times once a donor eligibility determination has been made (§ 1271.55). For such tissues, FDA compliance programme 7341.002 – Inspection of Human Cells, Tissues, and Cellular and Tissue-Based Products applies.

Each HCT/P that is manufactured must be assigned and labelled with a distinct identification code that relates the HCT/P to the donor, to all records pertaining to the HCT/P; and to the recipient. The code may not include an individual's name, social security number, or medical record number (§ 1271.290c).

Manufacturers must investigate any adverse reactions and deviations related to an HCT/P they made available for distribution. Reportable adverse reactions must be submitted to FDA within 15 days of receipt of information as a MedWatch Form 3500A. Adverse reaction means a noxious and unintended response to any HCT/P for which there is a reasonable possibility that it was caused by the HCT/P (Part 1271.3(y)) and deviations relate to events that represent a deviation from applicable regulations, standards or established specifications that relate to prevention communicable disease transmission (§ 1271.3(dd)).

[8] Currently at http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/EstablishmentRegistration/TissueEstablishmentRegistration/FindaTissueEstablishment/ucm110270.htm

44.4.1.4 Regulation of 'Manipulated' Tissue

Only very few cell therapy products are on the market to date, many products of relevance in this sector are currently in early stages of development. We will concentrate on the regulatory requirements for conducting clinical trials in this sector.

The Food, Drug and Cosmetic (FD&C) Act requires demonstration of safety and effectiveness for new drugs and devices prior to introduction into interstate commerce. The Public Health Service Act (PHS) requires demonstration of safety, purity, and potency for biological products before introduction into interstate commerce.

Consequently, pre-marketing authorization clinical studies must be performed under exemptions from these laws.

For drugs and biologics, a Investigational New Drug (IND) application must be filed (§ 312), for devices an Investigational Device Exemption (IDE, § 812).

44.4.2 Interactions with FDA

Once the responsible FDA division has been identified as outlined above, sponsors may take advantage of a pre-IND meeting opportunity to seek Agency guidance (§ 312.82). However, an important distinction should be made between 'official' and 'informal' pre-IND meetings. The Sponsor may request a formal pre-IND meeting from FDA which should be scheduled to occur within 60 days of FDA receipt of the meeting request. The former provides the investigators with formal advice that reflects 'current thinking' –it will subsequently be very difficult for the team to deviate from that advice without extremely good justification. Generally, FDA will not grant more than one pre-IND meeting for each potential application.

Informal advice can often be sought by interactions with the regulators.

44.4.2.1 Preclinical Data

The sponsor of a clinical trial should provide "…adequate information about the pharmacological and toxicological studies…on the basis of which the sponsor has concluded that it is reasonably safe to conduct the proposed clinical investigations." (CFR 21 Part 312.23 (a)(8)).

The kind, duration, and scope of animal and other tests required vary with the duration and nature of the proposed clinical investigations. Potential for tumorigenicity and the potential for inappropriate differentiation at a non-target location are significant safety concerns especially with hESC derived products.[9] Selection of the

[9] CTGTAC Meeting #45, April 10, 2008 Briefing: "Cellular Therapies Derived from Human Embryonic Stem Cells – Considerations for Pre-Clinical Safety Testing and Patient Monitoring".

most appropriate animal species and models is a major unresolved issue. In addition to the species used, the safety assessment of many cellular therapies has also made use of animal models of disease/injury that mimic some aspect of the pathophysiology of the proposed patient population. Such models help provide insight regarding dose/activity and dose/toxicity relationships. Thus, the applicability of such models in the context of species-specific immunology should be addressed. Cell survival, migration/trafficking, differentiation/mRNA or protein expression profile, integration (anatomical/functional), and proliferation also may need to be considered when selecting appropriate preclinical models.

44.4.2.2 Application

The contents of IND and IDE applications are similar. Beyond a description of the product and its manufacturing they will contain an account of preclinical studies including patient inclusion and exclusion criteria, study end points, patient follow-up, data monitoring and stopping rules. A list of standard operating procedures (SOP) will normally suffice for submission but critical SOP should be supplied in detail.

A Drug Master File (DMF) may be used to provide confidential detailed information about facilities, processes, or articles used in the manufacturing, processing, packaging, and storing of the product. Facility design and layout, production steps, contingency arrangements and personnel records must be relayed and may be referenced in a 'Type 5' DMF where such information already exists with FDA.[10]

Both IND and IDE investigations require Institutional Review Board (Ethical) assessment and approval. FDA must respond to the IND application within 30 days.

44.4.2.3 Phase 1

Phase I clinical trials (§ 312.21(a)) are typically designed to assess tolerability, or feasibility, for further development. In many situations, conducting the first-in-man study under an IND or IDE as a 'classis' phase1/feasibility study in healthy volunteers will be inappropriate for cell therapies.

FDA confirms: "We recognize that it may not be possible to follow each recommendation. For example, with some cellular products, it may be impossible to retain samples of the final cellular product due to the limited amounts of material available. Therefore, we recommend that you include your justification for adopting additional controls or alternative approaches to the recommendations in this guidance in the records on the phase 1 investigational drug."[11]

---

[10] http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122886.htm

[11] FDA Draft Guidance for Industry: INDs—Approaches to Complying with CGMP During Phase 1.

An investigational cell therapy for use in a Phase 1 study, is subject to the statutory requirements set forth at 21 U.S.C. 351(a)(2)(B). The production during Phase 1 is exempt from compliance with the cGMP regulations in part 211 but the exemption ceases if the investigational cell therapy has been made available for a Phase 2 or Phase 3 study or been lawfully marketed.

44.4.2.4 cGMP Criteria

The legislative framework for GMP requirements is set in §§210 and 211 for pharmaceuticals and for devices (Quality Systems Regulations) in §§820.

Staff qualifications and continued professional training need to be specified and recorded (§ 211.25).

Release criteria in 21 CFR 610 include sterility (§ 610.12) as common in biologics[12] including growth-promoting properties of the culture medium (note that some stem cell lines are reportedly compromised by mycoplasma[13]), identity (§ 610.14, established either through the physical or chemical characteristics of the product, inspection by macroscopic or microscopic methods, specific cultural tests, or in vitro or in vivo immunological tests) and potency – in vitro or in vivo tests, which have been specifically designed for the product so as to indicate its specific ability to effect a given result (§ 610.10; § 610.10; § 600.3(s)). This can be problematic in some cell therapies where the mode of action is a complex systemic interaction, but FDA representatives have given verbal indication of being alert to this complexity. Of particular interest for advanced therapies are the stipulations on culture (§ 610.10; § 610.18) which must be stored by a method that will "retain the initial characteristics of the organisms" – this obviously has to be reconsidered for complex cell derivation protocols. Moreover, the regulations talk about "source strains" and "seed lots" – which equates to 'master cell banks' and sub-cultures. Periodic tests must be performed to verify the integrity and purity of the culture and these results must be recorded and retained (§ 211.188; § 211.194).

Cell lines used for manufacturing biological products shall be:

(i) Identified by history;
(ii) Described with respect to cytogenetic characteristics and tumorigenicity;
(iii) Characterized with respect to in vitro growth characteristics and life potential; and
(iv) Tested for the presence of detectable microbial agents.

These rules do not apply to primary cell cultures that are subsequently subcultivated for only a very limited number of population doublings.

[12] Pharmaceutical Inspection Co-operation Scheme. Recommendation on sterility testing, Pharmaceutical inspection convention (1 November 2002) PI 012-1.

[13] Cobe et al. (2007) Microbiological contamination in stem cell cultures. Cell Biol Int 31(September)991–995.

FDA recognised that investigational cell therapy products may be manufactured as one batch per subject,[14] nonetheless testing of each batch for viability, cell number, mycoplasma and endotoxins close to the moment of application will usually be expected. Moreover, regulators ask for metrics on identity and potency but it is recognised that these may be 'moving targets'.

44.4.2.5 Expeditions and Facilitation

There are ways to expedite the process: Firstly FDA will 'fast track' the review if the product is intended for the treatment (or, in the case of devices, diagnosis) of a serious or life-threatening condition and it demonstrates the potential to address unmet medical needs.

When there is sufficient clinical experience to establish the safety of a product after use outside the US or in a different patient population, the FDA may review data from clinical studies performed outside the US in both the IND/IDE application or in an application for marketing approval. For devices, the sponsor can demonstrate substantial equivalence of the device to a legally marketed predicate device (510(k)).

Another way to speed up the process is to gain a Humanitarian Device Exemption (HDE) for certain devices (FD&C Act, §520 m) or an orphan drug designation (FD&C Act, 525, et. seq.). A device may be marketed under the humanitarian exemption for treatment or diagnosis of a disease or condition that affects fewer than 4,000 individuals per year in the US. The exemption relates to the effectiveness requirements for devices (FD&C Act, 529(m)(1), as amended February 1998). Several engineered skin constructs have been approved for market under the humanitarian exemption. Orphan drugs are those intended to treat a disease or condition affecting fewer than 200,000 individuals in the US for which there is little likelihood that the cost of development will be recovered from sales in the US. Other benefits of an orphan drug designation include grants and tax credits for clinical trials, FDA fee waivers and marketing exclusivity in the US for a period of 7 years from the date the compound is approved.

44.4.2.6 Vigilance

Manufacturers and clinicians should report adverse events through the FDA 'MedWatch' process. Post marketing studies may be a condition of the FDA approval, which may often be the case for novel cell therapies. Devices manufacturer may be required to conduct postmarket surveillance for any device which is a class II or class III device the failure of which would be reasonably likely to have

[14] FDA Guidance for Industry CGMP for Phase 1 Investigational Drugs; July 2008.

serious adverse health consequences or which is intended to be implanted in the human body for more than 1 year, or for a life sustaining or life supporting device (SEC. 522. [21 USC § 360 l]).

44.5 Regulatory Policy

It is implicitly clear that all discussions on 'Advanced Therapies' concern regimes of scientific, clinical and commercial conduct that do not fit the mould of existing medicines.

- Advanced therapies depart from a focus on 'simple' ligand-receptor interactions, but often also do not present a product the effect of which can be defined purely by its presence (such as whole-organ transplantation). Both safety and functionality of the product cannot be assessed with any significance in vitro.
- Cells and tissues are very complex entities that react very sensitively to a variety of stimuli, some of which cannot be replicated in vitro. Cell populations in many therapies are necessarily heterogeneous. The search for optimal purification protocols which is applicable for other contexts may not be appropriate for ATMP.
- Stem cells are often used precisely for their ability to differentiate into a variety of cell types and to engender changes in surrounding tissue. Thus any isolated assessment of proliferation profile and reactivity will always be insufficient. Almost all cells harbour a potential to proliferate in unexpected ways.
- Whereas in established 'pill-type' and biologics manufacture large 'lots' and 'batches' are released and tested, ATMP are often produced specifically for a particular patient. This means that regulatory provisions on product release testing may not only be inappropriate but also create a disproportionate burden.

44.5.1 Different Protagonists

Clinical trials require in-depth discussions between manufacturers, clinicians and regulators. Traditionally, only large pharmaceutical companies are equipped to shoulder the burden of maintaining GMP manufacturing facilities, of coordinating complex trials to the requisite standard and to meet the considerable bureaucratic requirements. The European ATMP regulation established special provisions and cost benefits for SMEs. However, it is sometimes overlooked that a great proportion of ATMP are not pioneered by industry but as individual ventures at a single (university) hospital, often on the initiative of clinicians collaborating with local academic groups.

It has been observed that for many ATMP products, especially cell-based and patient-specific treatments, the pharmaceutical industry has limited interest (and know how) in playing its 'usual' role of financing development and of acting as a sponsor in clinical trials.

Several reasons have been suggested:

- Many ATMP are manufactured very differently from mainstream medicines.
- It is often not possible to conduct trials on a large patient population.
- Many ATMP are seen to be more closely related to transplantation, an area that does not interface much with industrial R&D.

In many instances, the 'spin out' of ATMP development from the academic GMP facilities also meets technical difficulties: Purification of a specific ATMP product requires a highly specialised skill mix which combines elements of scientific expertise, with technical know-how and a strong clinical link to the treatment protocols of the individual patients.

Consequently, academic facilities are major contributors to the development of ATMPs. Not only do they have an important function in the translation of pre-clinical academic research into GMP, but many products may only reach clinical application by relying exclusively on academic facilities.

44.5.2 cGMP – Trying to Make Fit

Although the ATMP regulations are oriented towards the granting of a marketing authorisation its reach does not just extend to the 'launching' of a finished product on the Common Market. As we have seen, regulatory stipulations apply to every stage of development in clinical trials and even reach into pre-clinical development. One effect of the recent regulatory initiatives is to extend considerations of GMP to the area of ATMP. Rules on GMP have evolved over decades to ensure standards of quality, safety and efficacy in the development of pharmaceutical products. They stipulate a 'clean room culture' where every step is carefully monitored, controlled, validated and recorded. It is universally acknowledged that established GMP standards cannot simply be imposed on cell therapies without modification.

In fact, the ATMP Regulations in Europe were partially created to address this issue, but judging by the picture that emerges in this area, there remains a real concern that standards and practices in other fields are imported and imposed to advanced cell therapies without a careful assessment of whether these standards are appropriate and effective.

44.5.3 Some Examples

44.5.3.1 'Biomolecules'

The revised Annex 4 of 2001/83/EC contains the innocuous sounding provision: "For somatic cell therapy medicinal products and tissue engineered products, producing systemically active biomolecules, the distribution, duration and amount of expression of these molecules shall be studied." (4.3.2b)

This requirement could be interpreted as putting an unwarranted and unobtainable burden on complex ATMP. As an analogy: in organ transplantation, the 'biomolecules' emitted by the whole organ are not generally studied let alone exhaustively understood. Anyone familiar with recent scientific discourse in cell therapy will recognise that some perspectives are ascribing therapeutic benefit to the systemic interactions that the cell therapy induces, rather than to particular functions of the transplanted cells in situ. The mission to chart in detail every 'systemically active biomolecule' that a cell may produce in vivo is one that may well occupy generations of scientists for decades. The requirements of this provision could be seen to depart from the risk-based approach that the regulations posit.

44.5.3.2 Tumorigenicity

An issue that has created a great deal of concern for regulators in the US is the proposition of using cells with a multipotent differentiation profile, as such cells may 'revert' and grow uncontrollably in the recipient.

Where tumorigenicity is a theoretical concern, it is necessary to validate these applications using animal testing and ultimately in clinical trials. It is worth bearing in mind that decades of stem cell transplants have not produced large scale incidences of cancer. In situations where ATMP represent the only option to halt or mitigate the progression of a serious life threatening condition, lingering concerns about the long-term neoplasia risks must be weighted against a patients chances of survival without the intervention.

44.5.3.3 Hospital Exemption

It is clear from the proceedings that led to the Advanced Therapies Regulations, that the issue of a 'Hospitals Exemption' involved protracted discussion among Member States with very different positions and perspectives. The current wording therefore represents a baseline consensus, from which Member States are called to develop their own regimes. The context developed here is one that seeks to protect patients, but acknowledges that particular types of bespoke treatment are firmly a category apart.

The law support a clinician's unique right and responsibility to determine the best course of treatment for an individual patient. Many unlicensed stem cell therapies are only an option in very seriously debilitating or life-threatening conditions where no effective, licensed treatment alternative is available. In such cases, regulators must not encumber the decision making process but instead facilitate it by providing guidance about the management of situations in observance of appropriate safety standards.

Thus, where Art.3 (7) 2001/83/EC as amended states "Member States shall ensure that national traceability and pharmacovigilance requirements as well as

the specific quality standards referred to in this paragraph are **equivalent** to those provided for at Community level in respect of advanced therapy medicinal products for which authorisation is required" (our emphasis) we suggest that the level of equivalence required is approximate similarity, not one of identical application. While it is not yet clear how these requirements will be interpreted across Europe, what we have seen emerging in the UK and Germany may give rise to concern, where they allow no leeway on GMP standards. The aim of charting and enforcing (current) Good Manufacturing Practice is to operate high standards in the production of medicinal product. Often these regimes aim at establishing protocols that are robustly applicable in defined conditions over extended periods of time and generate products of comparative makeup, for example to avoid batch inconsistency. Where a treatment is inherently "non routine", these considerations of GMP are not as pertinent. For example, whereas GMP assessments focus strongly on Standard Operating Procedures (SOP), there is, by definition, no "standard" in non-routine ATMP production.

A further complication is introduced by the fact that data generated by relying on the Exemption cannot be generated for the purpose of analysing it scientifically – otherwise the treatment may be considered a clinical trial, which introduces a new set of regulatory requirements. This is clearly at odds with much of the discussions surrounding the exemption where it was assumed by many parties that small scale, proof of principle trials could be conducted under the exemption.

44.6 Summary Outlook

When considering the regulatory approach to novel cell and tissue based therapies, regulators in both Europe and the US have embarked on a precarious road: on the one hand copious new regulatory provisions and guidance suggest that the Regulators are responsive to the different nature of these therapies and aware of their potential. On the other hand there is a clear tendency to make the new regimes fit the existing mould as much as possible. In the US, greater discretion seems to be left with the regulatory authorities whereas in Europe the approach is officially more text-based while it remains uncertain how specific provisions will be interpreted by assessor and inspectors for EMEA and at Member State level.

By 30 December 2012, the European Commission shall publish a general report on the application of the ATMP-Regulation. By then it may already become apparent whether the regulatory approaches are sustainable or whether the concerns of inappropriate regulation we have alluded to here are justified. Until then, researchers, clinicians and entrepreneurs pioneering regenerative medicine treatments are in the position of not just scientific but also regulatory trailblazers – at dire risk of colliding with emerging rules but also with an opportunity to shape regulatory attitudes and regimes. In turn, regulators must be aware that in an emerging field even

'little things' such as inability to access appropriate guidance or rigid application of inappropriate standards can have an instant 'ripple' effect on the entire fledgling community and can inadvertently stifle all innovation at least in that particular branch of regenerative medicine.

Acknowledgements We are grateful to Dr. Anke Wagner (RTC Rostock), for her expert advice, craftsmanship and patience in designing the illustrations in this chapter.

Chapter 45
Ethics and Law in Regenerative Medicine: A Legal and Ethical Outline on Regenerative Medicine in Research in France, Germany and Poland

Gregor Becker and Anna Grabinski

Abstract Although France, Germany and Poland share common legal roots, the national laws of biomedicine distinguish themselves. Working out these differences in existing law the picture drawn shows how particular the law situation in each of the countries is and that these differences are caused by basic different ethical adjustments.

The specific relation between ethics and law in general and in particular in the three neighboring countries has finally led to different procedures to deal practically with research in the Regenerative Medicine, described and critically evaluated in the following.

45.1 Law Part

France, Germany and Poland are three countries with written laws that share common legal roots. Generally speaking, and especially in France and Poland, many similarities can be found between legal answers, for instance within civil or consumer laws. However, if there is one area of law where each of these European countries distinguishes itself, it is that of biomedicine.

The three countries we will study are members of the European Union and have recently adapted the European standards relating to the main activities within biomedicine such as clinical drugs trials as well as standards relating to organs, tissue

G. Becker, Ph.D. (✉)
Group for Bio-Ethics in Life Sciences (GBLS), Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, ul. Gronostajowa 7, Kraków 30-387, Poland
e-mail: gregor.becker@uj.edu.pl

A. Grabinski
Paris, France
e-mail: anna.grabinski@gmail.com

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_45,

and cells. In addition, the three countries have all signed the Oviedo Convention[1] and are on track to ratify it.

However, it is notable that on the subject of bioethics, the differences in legal solutions implemented by these countries are as numerous as the points they have in common: European standardisation does not infer an obligation to accept and legitimise biomedical practices and activities themselves. Rather, member states alone hold the power to make decisions with respect to the authorisation or banning of biomedical activity.

So this decision, the choice of whether or not a practice is acceptable, is not for the law to determine, rather the laws are merely a formal expression of country's values and beliefs.

In the area of biomedicine, perhaps more than any other, legal ruling is dependent on the ethical, cultural, historical and/or religious beliefs of a country.

The framework of research into regenerative medicine provides an excellent example of this.

45.1.1 Regenerative Medicine and Research

45.1.1.1 Regenerative Medicine and Fundamental Research

Embryonic Stem Cell Research

Without a doubt, human embryonic stem cell research gives rise to the greatest amount of scrutiny and questioning as it involves destroying the embryos.
Whatever the nature of this questioning (religious, ethical…), it leads to certain unease when linked to the human aspect of the embryo.

Moreover, it is this human aspect that generates the well-known question asked by jurists from countries whose legal rules are the legacy of Roman law: is the embryo a person or a thing?

The experimentation on an embryo with unmistakeably 'human' characteristics, generally considered as a potential human being,[2] gives rise to the fear that human exploitation will become accepted.

It is therefore no surprise to find a systematic ban on embryo research, as well as protective legal guidance in countries which have already adopted legislation relating to embryo research.

---

[1] Council of Europe. Convention for the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine: Convention on Human Rights and Biomedicine. Oviedo, 4.IV.1997 Available for consultation on: http://conventions.coe.int/Treaty/en/Treaties/Html/164.htm

[2] That which 'would ensure the widest ethical consensus' according to the French Council of State. Further reading: Study – "The revision of bioethical laws", Study adopted by the General Plenary Session, Les études du Conseil d'Etat, 2008, p. 12.

This is the case in Germany and France, however, not all European countries have followed suit. For example, the legal situation is more complex in *Poland* which has not yet adopted specific legislation for embryonic research.

Embryonic Stem Cell Research in Germany and France: A Unique Case

Germany[3] instituted a law protecting the embryo early on in 1991. In accordance with this law, the human test tube embryo cannot be the object of any research unless the research relates to the embryo's own protection.

Nevertheless, on June 28, 2002, the Bundestag passed a federal law on stem cells (Stem Cell Act – 'Stamzellgesetz'- StGZ) through which it authorised research on imported embryonic stem cells.

This law was very restrictive as it states that research can only be carried out on stem cells obtained from outside German territory prior to January 1, 2002.

On April 11, 2008, the StGZ law was modified by a slight easing of the conditions. It now authorises research on descendant stem cell lines taken from embryos obtained outside German territory before May 1, 2007.

Apart from this exception, no major changes[4] have been brought to this law; research on surplus German embryos remains illegal.

In France, the issue of embryonic research is controlled by clauses in Law No. 2006-800 of August 6, 2004, relating to bioethics whose measures have been

---

Available for consultation on: http://www.conseil-etat.fr/cde/media/document//etude-bioethique_ok.pdf (text in French)

"The Council of State advises the Government on the preparation of bills, ordinances and certain decrees. It also answers the Government's queries on legal affairs and conducts studies upon the request of the Government or through its own initiative regarding administrative or public policy issues.

The Council of State is the highest administrative jurisdiction – it is the final arbiter of cases relating to executive power, local authorities, independent public authorities, public administration agencies or any other agency invested with public authority.

In discharging the dual functions of judging as well as advising the Government, the Council of State ensures that the French administration operates in compliance with the law. It is therefore one of the principal guarantees of the rule of law in the country.

The Council of State is also responsible for the day-to-day management of the administrative tribunals and courts of appeal.

Every year, 110 bills, 900 drafts of decrees and 3,000 non-statutory texts are examined by the Council of State" (http://www.conseil-etat.fr/cde/en)

[3] For further general reading on this point, see: Christian Starck, Embryonic Stem Cell Research according to German and European Law, 41 EUROPARECHT 1 (2006) Available for consultation on: http://www.germanlawjournal.com/pdf/Vol07No07/PDF_Vol_07_No_07_625-656_Articles_Starck.pdf

[4] For a summary of the debates and issues of the revision of the StGZ, see: http://www.etatsgeneraux.fr/base_documentaire/international/allemagne_bioethique.pdf (text in French).

For a complete study, see the opinion "Should the Stem Cell Law be amended ?" of the German National Ethics Council: http://www.ethikrat.org/_english/publications/Opinion_Should_the_Stem_Cell_Law_be_amended.pdf

codified within the Public Health Code. The principle of the ban on embryonic research[5] is stated in the first paragraph of Article L. 2151-5 of the Public Health Code in unequivocal terms: 'Human embryo research is prohibited.'

Nevertheless stem cells offer the hope of discovering new cures and as a result, the law of August 6, 2004 was modified on February 6, 2006 allowing for the possibility of embryonic research[6] for the next 5 years following the executive order issue.[7]

Similar requirements, and in both cases cumulative ones, govern ***German and French stem cell research***.

In France, as well as in Germany, human embryonic stem cell research can only be carried out on so-called 'surplus' embryos – those obtained through medically assisted reproduction but no longer required for the parental procedure.

Moreover, whether the embryos have been imported (Germany and France) or not (France only), the consent of both parents must be obtained prior to beginning research. (In France consent must be obtained twice with a 3 month interval between each affirmation) The embryos cannot be exchanged for money and, if imported, they cannot be obtained by violating any laws in place in the exporting country.

Finally, in order for the research to obtain legal authorization from the relevant authorities (the Robert Koch Institute in Germany and the Biomedicine Agency[8] in France[9]), the research must be likely to allow major progress in treatments and also

---

[5] The human embryo is further protected with regulations more far-reaching than the banning of research. Articles L.2151-1 to L.2151-4 in fact state other bans:

That of **cloning**, whether it be reproductive, for therapeutic purposes, for research purposes or for use in commercial or industrial purposes

The creation of embryos for research purposes as well as industrial or commercial purposes

The use of the embryo for commercial or industrial purposes

[6] Just as OPECST sums up perfectly in its report "In the beginning, Article 37 of the 2004 Law gave ministers of Health and Research the power to authorise the importation of embryonic stem cells, provisionally, after a recommendation by an ad hoc committee (decree No. 2004-1024 of September 28th, 2004; finalised on September 28th, 2004). These measures were intended to allow French researchers to answer calls for projects launched by the European Commission. The law was then supplemented by the decree No. 2006-121 of February 6, 2006 relating to embryo and embryonic stem cell research and modifying the Public Health Code. This exceptional arrangement actually began on February 6, 2006 and is due to run until February 5, 2011." Report on research into the functioning of human cells. Report No. 3498, AN, drafted by M. Alain CLAEYS. Available for consultation on: http://www.assemblee-nationale.fr/12/rap-off/i3498.asp (text in French).

[7] Decree No. 2006-121 of February 6, 2006 relating to research on the embryo and embryonic cells and modifying the Public Health Code, JORF No. 32 of February 7, 2006 page 1974.

[8] Following the creation voting of the French Bioethics Law of August 6th 2004, the Agence de la biomédecine is the only such public body in Europe to combine the four domains of organ procurement, procreation, human embryology and genetics. Its close links with medical teams and patients enable it to ensure respect for safety and quality, anticipation, ethics and transparency". (http://www.agence-biomedecine.fr/agence/english.html)

[9] Just as Article L. 2151-5 of the Public Health Code sets out in paragraph 5: "The decision to authorise is taken according to the scientific relevance of the research project, its terms of implementation from the point of view of ethical principles and its interest to public health. The agency's

be the only way of obtaining the expected results, which is only possible if no alternative and comparably effective method exists.

Research on cells obtained from embryos or foetuses originating from pregnancy terminations can be carried out in France and is subject to laws relating to the removal of tissue and cells (Article L. 1241-5 of the Public Health Code). After the woman whose pregnancy has been terminated has been informed of the purpose of the removal, her written consent is obtained. Research protocol must first submitted to the Biomedicine Agency and can be suspended or banned by the Ministry of Research when the scientific relevance or the need for the removal is not established or when respect for the ethical principles is uncertain.

Germany only allows embryonic stem cell research on surplus embryos obtained from medically assisted reproduction. Therefore the issue of using foetuses or embryos from terminated pregnancies does not arise.

Can We Expect Legislative Changes?

That is the question being asked in France at the moment. In fact, Law No. 2004-600 of August 6, 2004 will be revised very soon.[10]

Many reports, drawn up by the main institutions in charge of these issues (the Biomedicine Agency and its advisory council,[11] the Parliamentary Office for Scientific and Technological Options,[12] the Council of State…), have been published or are being compiled, and many key figures have been or are being consulted.

Furthermore, General Estates on Bioethics have been put together so as to allow members of the public to express their opinions on the group of subjects relating to bioethics. The summary of the final report[13] on this public consultation was submitted to the President of the Republic on July 16, 2009.

---

decision, accompanied by advice from an advisory council, is communicated to ministers responsible for Health and Research who can, when the decision authorises a factual record of observations, ban or suspend it from being carried out when its scientific relevance has not been established or when respect for the ethical principles is uncertain."

[10] Ever since their first issues, France chose to review bioethics laws on a regular basis, so regulations remain consistent with fast scientific evolutions and improvements. This policy is also an effective way to address outdated measures. This revision is theorically intended to be set every 5 years. After a first late revision (only settled in 2004), the next revision should be conducted with, more or less, respect to the deadlines, since it is scheduled for 2010.

[11] The advisory council of the Biomedicine Agency brings together experts in the fields of science, medicine and human sciences, representatives of associations and various institutions, and members of Parliament. It watches over the consistency of the Agency's actions as well as the respect for statutory and ethical principles applicable to these activities.

[12] Parliamentary Office for Scientific and Technological Options, "Report on the evaluation of the appliance of the bioethics law of August 6, 2004". Report No. 1325 (Assemblée Nationale) and No. 107 (Sénat), drafted by M. Alain CLAEYS and M. Jean-Sébastien VIALATTE. Available for consultation on: http://www.senat.fr/rap/r08-107-1/r08-107-11.pdf (text in French).

[13] See: http://www.etatsgenerauxdelabioethique.fr/uploads/rapport_final.pdf (text in French).

The question of embryonic research continues to cause controversy: should we return to an absolute ban, as was the case when under the rule of the bioethical laws of July 29, 1994? Should we keep the current solution of compromise, namely a temporary opening? Or, should we proceed with a continued opening and give wider and/or consistent authorisation to embryonic research?

The Council of State[14] assessed authorized research results as well as the current body of knowledge regarding stem cell research and has declared itself in favour of embryonic research. It is the case that today's embryonic stem cell research and adult stem cell research compliment each other; therefore, there is a real scientific justification for pursuing stem cell research.[15]

According to the Council of State, continuing temporary exemptions cannot be justified, as there is no reason to believe that embryonic research will no longer be of scientific interest in years to come. The Council suggests dropping the blanket stem cell research ban by creating and implementing a permanent but strictly controlled system of stem cell research authorisations. This would be a straightforward right of the Biomedicine Agency, but not a duty to deliver it.[16]

This is equally the position of the Office for Evaluation of Scientific and Technological Options, which is in favour of a supervised system of authorisation.[17]

This issue of a new system of embryonic research is also raised by ***the Biomedicine Agency***. The Agency encourages legislators to consider many points when discussing future laws relating to bioethics. In particular, there is the issue of nucleus transferral, the possibility of creating embryos for research or even the creation of biological resource centres allowing the preservation of embryos for research without a specific aim.[18]

In this respect, the Advisory Council[19] questions not only the requirement for 'major progress in treatment and cure' in particular in the widening of its scope to 'scientific and medical progress', but also making the authorisation of research subject to there being an absence of effective alternatives, pointing to the issue of cells resulting from nucleus transfer as well as the need to resort to surplus embryos which have not been created for research.[20]

---

[14] See above, note 1.

[15] See: Study of the Council of State – "The revision of bioethical laws", p. 18.

[16] For further developments, see: Study of the Council of State – "The revision of bioethical laws", pp. 12–27.

[17] See: Parliamentary Office for Scientific and Technological Options, "Report on the evaluation of the appliance of the bioethics law of August 6, 2004". Report No. 1325 (Assemblée Nationale) and No. 107 (Sénat), drafted by M. Alain CLAEYS and M. Jean-Sébastien VIALATTE, p. 193 Available for consultation on: http://www.senat.fr/rap/r08-107-1/r08-107-11.pdf (text in French).

[18] See: Biomedicine Agency Report, Application Report – Bioethics Law of August 6, 2004, Report to the Minister of Health, Youth, Sports and Associative Life, October 2008, pp. 58–80 http://www.agence-biomedecine.fr/uploads/document/rapport-bilan-LB-oct2008.pdf (text in French).

[19] See above, note 8.

[20] For all these questions, see: Advisory Council, Contribution of the Agence de la Biomédecine Orientation Council pertaining to the bioethics law revision preparatory debates . Lessons of experience (2005–2008) and Questionings, pp. 30–41 http://www.agence-biomedecine.fr/uploads/document/bilanLB-partie3.pdf (text in French).

In the end, the result of the final report[21] on ***The General Estates on Bioethics***[22] is that it opens the door to criticize the need of a mere 'therapeutic aim' for embryo research and embryonic stem cell research. The report suggests that allowing research with therapeutic aims could justify the authorisation of research which might actually be unethical.[23] Furthermore, the term 'therapeutic aim' indicates simple potential consequences of a piece of research, whereas, by definition, the research is already laden with uncertainties, including the nature of the knowledge it will bring to light. That being the case, just as the Advisory Council of the Biomedicine Agency recommends, it would be preferable to replace the term 'therapeutic aim' with 'medical purpose' or even 'scientific purpose'.

The French public has also wondered about the changes in the legal system surrounding embryonic research and whether or not to maintain an automatic stem cell research ban accompanied by exemptions.[24]

The ***parliamentary proceedings*** are expected to begin in the first half of 2010 and it is very probable that a permanent and conditional authorisation will be the retained solution concerning embryo research and embryonic stem cell research.

Embryonic Stem Cell Research in Poland

The modified legislation of July 1, 2005 on tissue does not apply to embryonic cells.[25]

The proceedings and debates are under way, however, with the upcoming ratification of the Oviedo Convention.[26] This will also be an opportunity to reach a decision on the overall problems relating in particular to medically assisted reproduction, and with this, to legislate this point as well as embryo and embryonic stem cell research. For the time being, the legal framework for embryo and embryonic stem cell research must be analysed with regard to laws and regulations already in place.

- The absence of a clear legal solution in current laws and regulations

[21] See: http://www.etatsgenerauxdelabioethique.fr/uploads/rapport_final.pdf (text in French).

[22] For this expression of opinion and the issues debated on this occasion, see: opinion No.105 of the National Consultative Committee on Ethics. Available for consultation on: http://www.ccne-ethique.fr/docs/avis105anglais.pdf (text in English).

[23] See: Final report of The General Estates on Bioethics, p. 29 Available for consultation on: http://www.etatsgenerauxdelabioethique.fr/uploads/rapport_final.pdf (text in French).

[24] See: Final report of The General Estates on Bioethics, pp. 31–32 Available for consultation on: http://www.etatsgenerauxdelabioethique.fr/uploads/rapport_final.pdf (text in French).

[25] This law was revised on July 17, 2009 to conform with the Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells.

Available for consultation on: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:102:0048:0058:EN:PDF

[26] Council of Europe. Convention for the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine: Convention on Human Rights and Biomedicine. Oviedo, 4.IV.1997 Available for consultation on: http://conventions.coe.int/Treaty/en/Treaties/Html/164.htm

Dignity, deserving of respect and legal protection, is a principle inscribed in Article 30 of the Polish Constitution, which furthermore guarantees the protection of each man's life.

The Polish Republic also guarantees the protection of a child's rights. Thus, the law of January 6, 2000 has appointed an Advocate of Children's Rights. Within this law, the child is defined in Article 2.1 as: "a complete human being from its conception to its coming of age.[27]"

The legislation of August 30, 1996[28] inserted a second point into its first article: in accordance with this law, 'the right to life, including the antenatal phase, is subject to protection within the boundaries specified within the present law', thus modifying the law of January 7, 1993, relating to family planning, protection of the human foetus, and the conditions of admissibility for a pregnancy termination.

Moreover, it is because of the measures in this legislation that the Constitutional Tribunal had the opportunity to give a verdict on the matter of the dignity of the conceived child. This law, in fact, used to allow a pregnant woman, who found herself in difficult living conditions or in a difficult personal situation, to have recourse to a pregnancy termination.

In a judgement on May 27, 1997,[29] the Tribunal concluded that the Constitution was not in agreement with this clause. In fact, as far as it is concerned, the Constitution guarantees to protect human life throughout all stages of its development.

Meanwhile, in so far as the 1996 Law and the Tribunal's decision only affect pregnancy termination and, as a result, the embryo *in utero*, this raises questions as to whether or not the test-tube embryo benefits from the same protection and the same guarantees.

If the answer is assumed to be yes, test-tube embryos could be put in the same category as 'the conceived child', on which no scientific research is possible (unless undertaken in its interest), in accordance with the 3rd point of Article 26 in Chapter 4 of the law pertaining to the medical profession, relating to medical research.

Taking all these elements into consideration, there are two possible interpretations. The first is that combination of laws set forward would, in the absence of an exemption law, place an obstacle in the way of all human embryo research, even test-tube embryos.[30] On the other hand, a second interpretation could consider that the embryo is actually benefiting from a source of protection, which is not effective as long as there is no law to enforce it.

Consequently, since a law does not exist to regulate, surround, and, if need be, ban it, research on test-tube embryos and, therefore, on embryonic stem cells would

[27] According to Article 10 of the Polish Civil Code, the age of majority is conferred to the minor subject either when the minor reaches the age of 18 or gets married before the age of 18.

[28] Dz. U. 1996 r. nr 139, poz. 646.

[29] See: Constitutional Tribunal See: Constitutional Tribunal, 27.05.1997, *sygn. akt K 26/96* (OTK 1997, nr 2, poz. 19) (text in Polish).

[30] See in particular: A. ZOLL 'Can we talk about a legal statute on stem cells?' Gazeta Lekarska, No. 2005-02, Debata Available for consultation on: http://www.gazetalekarska.pl/xml/nil/gazeta/numery/n2005/n200502/n20050221 (text in Polish).

be possible. The two positions co-exist at the moment and only adopting a future law on Medically Assisted Reproduction will enable Poland to reach a final conclusion.

- Planned Legal solutions for the future law on Medically Assisted Reproduction

With the prospect of future legislation surrounding medically assisted reproduction six proposals[31] have been presented at Sejm, the Polish Chamber of Deputies to accompany the ratification of the Oviedo Convention.

Two of these proposals recommend a definite ban on all in vitro fertilisation; one was presented by the deputies from the presidential party, the PiS ('Prawo I Sprawiedliwosc' – 'Law and Justice'), and the other was the public proposal by the Committee of Legislative Initiative[32] 'Contra in Vitro'. It followed that all research carried out on embryos or on cells obtained from them would thus be banned.

On the contrary, two other proposals issued by the Prime Minister Donald Tusk's party were in favour of the administration of in vitro fertilisation, the conditions of which would be more or less restrictive depending on the project in question. Deputy GOWIN's proposal[33] suggests advocates for the banning of the preservation of surplus embryos as well as banning their destruction. Deputy KIDAWA-BLONSKA's proposal, on the other hand, accepts the cryogenic preservation of embryos and therefore the creation of surplus embryos.

However, the two proposals do have one point in common: by refusing to destroy embryos,[34] both of them make all cognitive projects, which are carried out using embryonic stem cells in particular, impossible to pursue.

A public proposal, following the initiative of the Federa Association (Federation for women and family planning),[35] advocates a wide opening for access to in vitro fertilisation. However, even though the proposal suggests that preserved gametes can be destroyed or used for research purposes when the person benefiting from this preservation makes such a request, such a possibility is not foreseen especially for embryos.

---

[31] On September 15th, 2009, date when the present text was written (author's note).

[32] Article 118 of the Constitution grants the right to legislative initiative to citizens, as long as the law proposal comes from a group of 100,000 citizens able to vote in legislative elections.

[33] Deputy GOWIN was named as head of the Bioethics Commission in 2007, in charge of the legislative proposal concerning the issues relating to in vitro fertilisation and supporting the ratification of the Oviedo Convention. The text of the proposal was fixed on December 6th, 2008. It is available for consultation on: http://ekai.pl/wydarzenia/x17354/co-dokladnie-mowi-projekt-ustawy-gowina-caly-tekst-projektu/(text in Polish).

[34] For the KIDAWA-BLONSKA proposal, see: http://www.platforma.org/pl/aktualnosci/newsy/art1488,kidawa-blonska-o-projekcie-ustawy-regulujacej-zasady-korzystania-z-metody-in-vitro.html (text in Polish).

For the GOWIN proposal, see Article 6: http://ekai.pl/wydarzenia/x17354/co-dokladnie-mowi-projekt-ustawy-gowina-caly-tekst-projektu/(text in Polish).

[35] Available for consultation on: http://www.federa.org.pl/Informacje/INF.pdf (text in Polish).

Nevertheless, Article 46a of the proposal holds that anyone who, against the wishes of the gamete donors, destroys embryos which could have been able to develop normally should be given a maximum 3-year prison sentence.

Two possibilities should be considered.

If the embryos are likely to develop normally, then their destruction may be ***carried out provided that it is the wish*** of the donors.

If, on the other hand, the embryos are unlikely to develop normally, then their destruction can be carried out without the donor's consent being necessary, or even in spite of the couple expressing their wish to the contrary. Consequently, embryonic stem cell research would be possible, despite the fact that it involves the destruction of embryos. It is worth noting that the proposal also makes provision for the banning of the creation of embryos for research, in accordance with the principle terms in the Oviedo Convention.

Unlike embryonic stem cell research, research on adult stem cells, a category which includes blood cells from the umbilical cord (also called placental blood cells), does not attract real controversy. Its importance is widely accepted and none of the three countries being studied are opposed to it.

Research on Adult Stem Cells

In France, the removal of tissue and cells as well as the harvest of human material from *living people* can be carried out for a scientific purpose (Article L.1241-1 of the Public Health Code). However, haematopoietic stem cells from bone marrow can only be harvested for a therapeutic purpose, as opposed to those from peripheral blood which can be harvested for scientific purposes.

The donor's consent must be expressed in writing after he/she has been "duly informed of the purpose of the removal or harvest and of the associated consequences and risks." (Article L. 1241-1 of the Public Health Code).

In certain cases, "when the nature of the removal and its consequences to the donor so justify" (article L. 1241-1 alinéa 2 *in fine*), rules relating to the donation of organs apply (Article L. 1231-1): the person will only give his or her consent after having been informed by the expert committee that the law makes provision for and before the presiding judge of the County Court or his representative or, in the case of extreme emergency, before the State Prosecutor.

Finally, neither tissue or cell removal nor the harvest of human material can be carried out on a minor or an adult who is under legal protection (for example, under supervision.)

'Operating remains', in other words the organs, tissue, cells and human material which have been removed during a surgical operation, can be used for research purposes, as long as the person does not object (Articles L. 1235-2 and L. 1245-2).

Removal for scientific purposes can also be carried out on *the deceased* (Article L. 1232-1).

For this to happen, the donor must not have expressed his or her refusal while alive, which must be listed in the National Register of non-donors.

However, in the case where the deceased person's wishes have not been expressed, the doctor must do his or her utmost to obtain from the next of kin the deceased person's possible opposition to organ donation expressed when alive and inform them of the intended purpose of the removal.

When the deceased is a minor or an adult under guardianship, the removal can only take place on condition that each of the holders of parental rights or the guardian gives written consent. However, if it is impossible to consult one of the holders of parental rights, the removal can take place on condition that the other holder gives written consent (Article L. 1232-2).

Removal for scientific purposes can only be carried out within the framework of agreements forwarded beforehand to the Biomedicine Agency (Article L. 1232-3).

The Agency must be notified of the removal before it is carried out (Article L.1232-1 final paragraph).

Lastly, "the minister in charge of research may suspend or prohibit the implementation of these agreements when the need for the removal or the relevance of the research has not been established." (Article L.1232-3).

Finally, it can be pointed out that Article L. 1211-2 of the Public Health Code provides the possibility of carrying out research on elements which have been removed for a given purpose (therapeutic or scientific), but to which a new aim can be assigned. French legislation makes provision for and allows these premises where "a change of purpose" is made. Only the person's lack of opposition is required then, after he or she has been informed of this other purpose. However, in accordance with this law, "it can be exempt from the obligation to inform if it proves impossible to find the person concerned again, or when one of the advisory committees for the protection of people mentioned in Article L. 1123-1, consulted by the person in charge of the research, does not deem this information necessary. these exemptions are not permitted, however, when the elements initially removed consist of tissue or germinal cells. In the case of the latter, any use other than the purpose for which it was initially removed, is prohibited in the event of the death of the person concerned."

In Poland, the removal of organs, tissue and cells is governed by the law of July 1st, 2005, modified by the law of July 17th, 2009.

Removal for scientific purposes can be carried out on *deceased people*. It is surprising to note that the conditions relating to the expression of wishes are only formally provided for in the premise of removal with a view to transplant. This oversight, moreover, was not corrected when the intervening law was modified on July 17th. 2009. Can we conclude from that that it is not necessary to seek the person's wishes? That strikes us as being contrary to the spirit of the law; we also think that the person's wishes must be sought whatever the purpose of the removal.

In accordance with Article 5 of the law, refusal must in fact be expressed while the person is alive and noted down in the Register of Non-Donors.

On the other hand, no provision has been made regarding removal of tissue and cells for scientific purposes from *a living person*.

It will be advisable then to consult common law rules which could be applied to the protection of the integrity of the human body.

Attacks on physical integrity receive penalties, which Articles 148–162 of the penal code[36] make provision for.

However, in accordance with Article 27 of the penal code, "a person is not committing an offence if he or she intends to carry out a piece of cognitive research (…), if the expected benefit is of essential cognitive, medical or economic importance, and if the hopes linked to the obtainment (the obtainment of the benefit), as well as the purpose and the way the research is conducted, are justified in the eyes of present medical knowledge."

What is more, the person's informed consent must be obtained: he or she must be informed beforehand of the expected benefits and the risks incurred, as well as the possibility of them occurring, and also that he or she can stop participating in the research at any time.

In Germany, a specific federal law relating to adult stem cell research does not exist; in fact, the law concerning stem cells only pertains to embryonic cells.

Therefore, it is necessary to look for possible rules controlling this type of research in the constitutional principles and general laws.

The freedom of research and of the profession of researcher is limited by the constitutional principle of the dignity of the human being and a person's right to self-determination. The latter results in as many constitutional principles (the right to life and physical integrity, the general right of the individual) as in common law, which prohibits attacks on a person's physical integrity without his or her informed consent.[37]

This principle of self-determination expresses itself therefore in ***the requirement of an informed consent***, prior to all removal of stem cells, whatever their nature and origin.

45.1.2 Regenerative Medicine and Applied Research

The use of adult stem cells in the context of research on human subjects implies that autologous or allogenic cellular products, which can sometimes be genuine patent medicines for which an authorisation will have to be requested to be put on the market, may be administered directly to a person.

In France, all research carried out on a person, whether or not it concerns health products, is subjected to a one single set of measures.

---

[36] See Chapter XIX "Offences against life and health" of the Penal Code.

Available for consultation on: http://prawo.money.pl/akty-prawne/ujednolicone-akty-prawne/kodeksy/kodeks;karny;z;dnia;6;czerwca;1997;r;,1997,88,553,DU,410.html (text in Polish).

[37] This requirement of collecting the informed consent can however be qualified when the research is intended on samples already in existence. As the German National Ethics Council specifies, the absence of the collection of further consent may, in certain cases, not constitute an illegal attack on the general right of the individual. It is mainly to do with premises where samples have been made anonymous. See the opinion "Biobanks for research" of the German National Ethics council: http://www.ethikrat.net/_english/publications/Opinion_Biobanks-for-research.pdf (text in English).

The rules surrounding this "biomedical" research appear in Articles L. 1121-1 and the those following of the Public Health Code.[38] These measures issue from the law No. 2004-806 of August 9th, 2004, which completely revised the modified law of December 20th, 1988[39] and was the opportunity to transpose the 2001/43/CE directive relating to clinical drugs trials.

All clinical research using adult stem cells are therefore subject to these measures.

In order for this to be implemented, the research protocol must also obtain the approval of the Committee on the protection of persons (Article L. 1123-7 of the Public Health Code) as well as the authorisation of the French Agency for the Security of Health Products (Afssaps)[40] (Articles L. 1123-8 and L. 1123-13).

Specific measures relating to clinical trials using embryonic stem cells do not exist. In the premise where a certain protocol had just been established, general rules such as the ones mentioned above would therefore be applied. However, in its report on the application of bioethical legislation,[41] the Biomedicine Agency makes it clear that after consultations with the agencies and the Ministry of Health, the obligation to take note of the advice of the ABM on research using differentiated cells made from human embryonic stem cells[42] has been proposed and could be confirmed during the future revision of the bioethical laws.

In Poland, research carried out on a human subject is governed by Chapter 4 of the Law pertaining to the medical and dentistry professions.[43]

So that the protocol can be implemented, the opinion of a Bioethical Commission is necessary, which expresses an opinion in the respect of the legal and ethical conditions of research. If it does not give its approval or it does subject to modifications, the promoter can submit his or her protocol to a Bioethical Commission of Appeal,[44] close to the Ministry of Health. An authorisation must also be granted by the Health Minister.

There is, however, a peculiarity relating to research concerning cell preparations. Put into the same category as pharmaceutical products, they are governed both by general measures relating to research on human subjects and also by the measures

---

[38] See: http://www.legifrance.gouv.fr/affichCode.do?idArticle=LEGIARTI000006685827&idSectionTA=LEGISCTA000006170998&cidTexte=LEGITEXT000006072665&dateTexte=20090818

[39] Law No. 88-1138 of December 20th, 1988, known as "the Huriet-Sérusclat law" relating to the protection of persons who participate in biomedical research, Official Journal of the French Republic (JORF), December 22, 1988.

[40] The French Agency for the Security of Health Products is in charge of a general task of evaluating the risks and benefits of health products. See: www.afssaps.fr

[41] The report is available for consultation on: http://www.agence-biomedecine.fr/uploads/document/rapport-bilan-LB-oct2008.pdf

[42] See: Biomedicine Agency Report, Application Report – Bioethics Law of August 6, 2004, Report to the Minister of Health, Youth, Sports and Associative Life, October 2008, p. 77 http://www.agence-biomedecine.fr/uploads/document/rapport-bilan-LB-oct2008.pdf (text in French).

[43] http://www.mp.pl/prawo/index.php?aid=40167 (text in Polish).

[44] http://www.kb.mz.gov.pl/index_en.html (text in Polish).

in Chapter 2 of the law "Pharmaceutical Law",[45] which sets out the conditions relating to the carrying out of clinical trials on health products.

The rules relating to research on human subjects are not the subject of general federal legislation ***in Germany***. On the other hand, research in certain types of activities or on certain products have been the subject of federal laws, such as, for example, the German Drug Act or also the Medical Products Act.[46]

As regards medicine, the jurisdiction to legislate on these points is shared with the Lander, which have, moreover, sole jurisdiction to establish the standards relating to the medical professions.

Therefore, in the absence of a specific federal law, measures can be found relating to biomedical research within the professional codes which, although established by professional orders, have legal strength.[47]

They contain, as is usually the case, the requirement for an informed consent from the person participating in the research, in accordance with the constitutional principles of dignity and self-determination, but also the need to submit the research protocol for the opinion of an ethical committee.

When the research is concerned with cellular products, it is, as in Poland, put into the same category as research on pharmaceutical products[48] and from then on, subject to one of the specific federal laws, the German Drug Act[49,50] whose measures relating to clinical trials transpose the 2001/20/CE directive of April 4th, 2001.

The German Drug Act defines thus the conditions for the implementation of clinical trials in Chapter 6, entitled "Protection of human subjects in clinical trials".

The opinion of an Ethical Committee (the Committee of the investigator's Land or, in the case of multi-centric research, that of the main investigator's Land) must be sought and the authorisation of the authority concerned, the Paul Ehrlich Institute,[51] must be obtained.

---

[45] http://www.mp.pl/prawo/index.php?aid=13358 (text in Polish).

[46] http://bundesrecht.juris.de/bundesrecht/mpg/gesamt.pdf (text in German).

[47] Just as Professor TAUPITZ mentions, a model of the professional code has been drawn up by the Federal Order of Doctors. It can therefore provide inspiration to the Lander in the drawing up of their own measures for professional codes. So, for example, Article 15 of the code model features the obligation to seek the opinion of an Ethics Committee before all research on a human subject. See: Jochen TAUPITZ, "Germany: current legislation" in Biomedical Research, Council of Europe publ., 2004, pp. 107–120

[48] They are specifically excluded from the field of application of the Medicinal products Act, Chap. 1, §2, pt. 5. See: http://bundesrecht.juris.de/bundesrecht/mpg/gesamt.pdf (text in German).

[49] On this point, see: SCHLENKE, TAPERNON et al., "The impact of the german tissue act on the manufacturing of autologous and allogeneic stem cell preparations", Transfus Med Hermother 2008;35:446–452.

[50] See the English version of the German Drug Law: http://www.bmg.bund.de/cln_110/nn_1200354/SharedDocs/Downloads/EN/health/AMG-pdf,templateId=raw,property=publicationFile.pdf/AMG-pdf.pdf

[51] See: http://www.pei.de/cln_109/nn_162554/EN/home/node-en.html?__nnn=true

45.1.3 Conclusion

Adult stem cell research, which the three countries studied agree is acceptable, is the area which shows the most similarities in the basic rules which surround it. It is hardly surprising and quite the norm in fact, to find two main legal –or rather, legal and ethical requirements: the respect for the requirement of informed consent and the need to seek the opinion of an ethical committee in the case of research on a human subject.

The questioning and hesitation surrounding the acceptability of embryonic stem cell research, the need for an ethical backing for its implementation, are themselves, without doubt, the most revealing of the close links between the law and the code of ethics.

45.2 Ethics Part

45.2.1 The Relations Between Ethics and Law

Although it cannot be the purpose of this article to provide a complete general outline on the nature of ethics, respective certain points have to be mentioned to regiment the importance of ethics into the current structure of the *ethical-legal systems* in Europe.

Generally, ethical guidelines seem to have a heterogeneous character.

The purpose of ethical guidelines is to provide concrete rules supporting and conducting respective decision making processes within a problem area and – *en passent* – already to represent a preliminary strategic policy regarding respective ethical discussions. The target group(s) of a guideline can vary immensely, as well as its impact.

Nevertheless – based on its nature -there is not even one ethical guideline that does not appear as a general binding declaration on its respective subject.

However, ethical guidelines are no instructions how to act in single cases, but just the middle-step of managing ethical problems – between existing law and concrete rules of action.

Main critics on European Ethics settings providing ethical guidelines – in what form ever – have to be formulated.

First of all, it appears that it has become not just fashionable to establish compliant ethics committees and commissions by governments, but that this fashion indicates the debatable political standing and importance of the "producers" of ethical guidelines. In this regard Krippner (2004) sees specific dangers connected: "Therefore, it may appear hardly odd to see the rising danger of a spreading "Expertocracy" or – a little malicious – to talk about the Renaissance of a "Soviet Republic"."[52]

[52] Krippner (2004), p. 239; translation from German to English by G. Becker.

Ethics in organised and often administrative form has developed a political standing that provokes criticism on several levels. Here and now, we only would like to mention the point that especially National Ethics Committees pretend representing the "nation's moral profile". It is questionable if it is at all possible to establish a Committee fulfilling this claim, but it is even more doubtful when such a Committee is established by political organs – and so the questions regarding the independence and whether such a Committee could be used as a "political toy" have to be raised.

Non-academic ethics is usually an ongoing evolutionary societal process and not a political action. To "extract" representatives from this process is at least a task that shall not be in the hands of politics. Nevertheless, the aim to organise ethics councils, giving a kind of bioethical outline is meritorious – against the background of a certain urgency to deal with bioethics.

Written statements of such committees are meant to be ethical guidelines for certain topics with – at least – national width.

The procedural connections between ethical guidelines and law making processes are in no case transparent. The concrete influence of guidelines being transformed to laws and regulations is unknown. However, using the example of the establishment of the German Stem Cell Act, it becomes clear that finally political thoughts, constitutional tactics, private attitudes and economic interests could prevail over pure ethical evaluations – and – that finally respective ethics committees and commissions fulfil – often unintended – a political role.[53]

Ethical guidelines rarely prove impact on rules of action – in default of rules of action![54] Rarely, ethical guidelines reach the facilities facing exactly the ethical problems the guideline deals with. Too often, they are acknowledged somehow, but keep abstract and disconnected or will be considered in administrative ways – form papers to fill out; however, the value oriented procedure of ethics commissions at work, resulting in ethical guidelines gets lost when they are transferred to law or to action rules. Furthermore, it must be said that biomedical institutions have a stronger interest in actions and results itself but acknowledging, developing and establishing concrete rules of action.[55]

---

[53] The question of the political state of ethical guidelines in many European States is *de facto* decided: the National Ethics committees, established – by what legitimation in this regard ever – by politics "furnishes politics a fit ethical occasion" by the ethical guidelines they prepare. It keeps debatable for pluralistic societies to disregard completely federal ideas of concrete ethics models, as e.g. represented by the Polish group for bioethics in Life Sciences: this pluralistic model follows the idea that the current top-to-bottom- model shall be replaced by a bottom-up model, in which Scientific institutions develop their own guidelines AND action rules – covered only by established law.

[54] In a way it is amazing that although bioethics has improved its importance in Europe over the last decade, "action rules" in form of "rules of conduct" in labs, sickrooms etc. have not been established. Bioethics has not reached (yet) the units where concrete work is done.

[55] Possible reasons for that kind of ignorance can be found in archaic self-images, in which Science itself has got an ethics *status* so developed that a concrete formulation on the "dos and don'ts" simply appears not necessary; another reason is the disability of Science and its representatives to deal concretely with that meta-level of Science without counsel – and in Europe there are not too many bioethics consultants with the ability helping implementation of concrete bioethics rules within Science.

It is in the nature of ethical guidelines that they often keep below – but never extravagate – the limits of existing law. Ethical guidelines use the leeway connected laws and regulations provide, and – *vice versa* – ethical guidelines, as moral workouts of normative character, influence law makers preparing laws. This way ethics develops political influence, although – as mentioned – it keeps absolutely unclear how this process works in details.[56] The correlation between ethics and law and the meaning of ethical guidelines within law-making processes neither follows fixed rules nor is the setting of involved parties determined. The question whether either level of ethics have any influence on law-making as a political process has – finally even – to be kept open. However, we claim that developing laws in pluralistic societies is always based on the consideration of respective ethics – here meant in a special way:

Ethics shall be the purposeful formulation of the current societal standing of moral attitudes of the respective society under consideration of the constitutional values of society. It is more but a moral "snapshot in time" or an ethical "cross-section" of ruling moral attitudes within a society:

For the final purpose of formulating a law, it considers the "moral pillars[57]" of society, formulated in the constitution.[58]

Based on that model of ethics, it is understandable that, e.g. the establishment of a modern abortion law in Germany, has become possible in the 1970, when it had been impossible in decades before; the ethical main stream in society does not only underlie evolutions, but also creates calls for action.

Furthermore, in reference to the German Stem Cell Act, as well as in reference to the German Abortion law, it is understandable that the formulation of both laws appear to be odd for non-lawyers. The consideration of the constitutional sacrosanct protection of life in principle has made it necessary in either case to use formulations – appearing as circumlocutions – explaining indirectly and in "bowed" ways the conformity with the constitution.[59]

---

[56] Finally, law makers in parliaments raise their hand pro or contra a law suggestion – what finally has moved them to vote in this or that way keeps their secret. However, hopefully they all have reflected ethically the law they vote on!

[57] Certainly, this is the "perfect case"; however, legal, political and tactical-political aspects are usually involved, too.

[58] In this context the motive of "eternal recurrence" is the question how to build up laws in a pluralistic society at all. The practical answer usually given is the hint to the formulas of basic rights fixed in the constitution. Such a "check-up" in practice can be extremely difficult when a problem touches from all possible sites different basic rights at the same time or when it touches ideas beyond law; the debates about the Stem Cell Act in Germany was such a case: apart from the right of "physical integrity" and "dignity of man", also the question of the beginning of Human life was relevant.

[59] The same is valid for example in German Medical Law. The circumlocution in this case is that a syringe given by a medic is an "assented personal injury" without criminal sanction or prosecution. This formulation has been necessary for the constitution includes the physical integrity (Grundgesetz Article 2), but syringing in the frame of medical treatment makes perfectly sense, too. So, the law-makers had to combine both circumstances in a formulation appearing slightly odd.

The described comprehension of ethics in practice counters definitions of ethics as discipline of Philosophy. Rules valid in Philosophy have no validity in the described setting. e.g. "*David Hume's guillotine*" and "*George Moore's naturalistic fallacy*", each describing on different levels the failure of logic methodology in philosophical ethics, regarding extrapolating the Good from described entities.

In Ethics in practice as described above, it is not only the case that *Moore*'s and *Hume*'s rules regarding ethics being independent from actual progression of Science have no validity; in Practical Ethics it is even the case that ethical evaluations **have** to be based on real existing circumstances and on facts, provided by Science.[60]

In regard of Stem Cell Research, the possible fact that only *so-called embryonic stem cells*[61] lead to concrete therapies is a strong and considerable argument supporting endeavours to justify morally Embryonic Stem Cell research.[62]

The involvement of Theology in ethics committees and commissions, preparing ethical guidelines for scientific work bears problematic aspects on different levels.[63] Briefly, the problems core lies in the question why church representatives shall be preferred to representatives of other societal groups when it comes to questions of Science and ethics.

However, a comparable bigger problem of the general setting of ethics committees is the kind of participation of law in ethics commissions, especially – like in Germany's Central Ethics Commission for Stem Cell Research – when the lawfulness of a research project has to be evaluated in addition to ethical aspects. Usually, like in the mentioned commission, a legal opinion by a specialist is essential for finalising the process of evaluation of the ethics commission.

Also on this level the question of the relation between ethics and law keeps still open. *Erwin Deutsch* formulates it this way: "The Ethics Commission ranks as an "Instance of Problematisation". Therefore, in difficult cases it appears recommendable that the commission bases its counsel on a legal opinion. One cannot hold against it that the legal opinion makes the work of the Ethics Commission redundant. Law

[60] This way it may be understandable that we call it debatable to involve ethicists from Philosophy in Ethics leading to concrete rules, regulation and laws. The aims of Philosophy, delivering a purposeless general outline of ethics is only in restricted ways compatible with the concrete goals of establishing rules. The consideration of e.g. Hume's and Moore's rules is – without any doubts" a "must" on the philosophical level of ethics, but contra productive and quixotic when it comes to societal relevance of real existing ethics, as a grown and developing societal phenomenon.

[61] Keller (2009) points out the debatable distinction of the distinctive terms "embryonic -" and "adult stem cells" against the background that these terms are not describing exactly "the source of withdrawal" in a biological and medical sense.

[62] Furthermore, it has to be said that there are certain indications that on a societal level the question whether it is morally justified to destroy Human Life in early stage for the benefit of sick people has found a clear positive answer. If it comes to the point that Embryonic Stem Cell research leads to life-saving therapies – becoming describable fact – societies may decide clearly for them, representing the ethical evaluation that these life-saving therapies have higher moral value but Human early-stage-life. This way, in real life, facts determine ethical evaluations, whereas the author disagrees on a moral level in the described case!

[63] The metaphysical argumentation of Theology might be incompatible in discussions of practical ethics. Furthermore, the point of secularisation is an open question in this context.

only represents the ethical minimum. Even a lawfulness research project can meet ethical concerns."[64]

Deutsch's phrasing that law only represents the ethical minimum hits the nail just half on its head – the ethical content of law is a "derivate" of the moral adjustment of the state under consideration of the current ethical status of society and the basic moral adjustment of the State expressed in the Constitution – and due to this context, law enjoys a lower "ethical concentration" than a pure ethical statement. However, disregarding this part of the nature of law, the meaning of law in the described context of an ethics commission cannot be overestimated: law builds the decision and working frame of an ethics commission. It is excluded that ethics commissions give counsel beyond existing law.

We find it an often disregarded aspect of ethics commissions that doing their work they are moving solely on legal ground: an ethics commission in the given frame is the exponent of law, meanwhile both – law and ethics – are either rooted in the ground of morality.

Nevertheless, this legal-bond of ethics commissions raises from one side the question of the independence of such a commission. From the one side the work of the ethics commission is framed by law, but we think that this does not speak against the idea of independence of ethics commissions in the way it is formulated in the "*Hong-Kong revision*" of the *Helsinki Declaration*. There it is described as "a specially appointed ethical review committee, which must be independent of the investigator, the sponsor or any other kind of undue influence".

Especially regarding ethical questions modern Sciences bring up, ethics commissions cannot afford working independently and unattached to law. Modern Sciences often touch aspects that enjoy constitutional protection. Therefore, the state has got a special interest in establishing ethics commissions and, we claim so, even a duty to take care for their establishment.[65]

In the following chapters we will give an outline on the respective situations of ethics committees in France, Germany and Poland.

Summarising, we already can mention that in these countries ethics is organised by politics.[66]

45.2.2 The Situation of Ethics Commissions in Poland

In Poland no official national ethics commission has been established yet. The Commission of Bioethics at the Medical Faculty of the University of Wroclaw is

[64] Deutsch (2006), p. 631.

[65] How far it is justifiable that a government or a ministry and NOT the parliament establishes ethics commissions, as it is the case in Germany referring the Central Ethics Commission for Stem Cell Research has to be released for further discussions!

[66] This is not a natural given fact. Over the years, inter alia the Polish Group for Bio-Ethics in Life Sciences has tried to convince Science to build up ethics unit as a "voluntary self-monitoring" to prevent a political takeover of bioethics as such; until now in vain…

mentioned in several lists of National Ethics Commissions, although officially it does not have this status.

Although in 2007 nominations for a National Ethics commission consisting of scientists, philosophers and priests were already sent to the candidates by the Polish government, an inaugural session has not taken place yet.

In Poland there are several ethics commissions, like the Advisory Council on Ethics in Research at the Ministry of Science and Information Technology, the Committee at the Presidium of the Polish Academy of Science, called "Ethics in Science", the Bioethics Appeal Committee at the Ministry of Health. Additionally, there are about 50 local research ethics committees and "Ad hoc committees", e.g. Stem cell research ethics committee at the President of the Polish Academy of Science.[67]

In April 2008, the Tusk administration has established the "Team for Biomedical Convention", consisting of well-known personalities from biomedical Science, Law, Catholic Theology and Philosophy; the ratio between these three groups keeps in equilibrium.[68]

Although, the task of this "Team" is the "analysis of the legal status in the field covered by the Biomedical Convention"[69] it has to be mentioned here. This "team" may develop importance referring national bioethics decisions (also connected with Regenerative Medicine) against the background of a missing National Ethics Commission.

It is probable that the "team's" decisions on legal regulation will anticipate ethical debates – not taking place yet. In this regard it can be claimed that the established "team" will take over tasks and will take decisions a National Ethics Commission shall be responsible for.

This way, sophisticatedly, the "team" seems to be a political instrument of the Tusk administration and is an evasion of the political difficulties connected with the establishment of a National Ethics Commission.

At the inaugural meeting of the "team for Biomedical Convention" Prime Minister Tusk stated truly in reference to legal backlogs in the field of legal decisions in their ethical and substantial aspects: "In this respect we are far behind the European Union member states."[70]

A second remarkable statement Prime Minister Tusk has made at the same occasion referring the political dimension of this group: "I hope that political interests will not disturb the intellectual and ethical dimension of this debate."[71]

---

[67] See also Szwarski (2004); the impact and tasks of the Ad-hoc commissions keep unclear. Generally, the state of sources referring ethics commissions and/or Regenerative Medicine are not satisfying. Statements and publications on officially planned actions referring law and ethics of Regenerative Medicine confine themselves on generalities or press statements without official character.

[68] See http://www.kprm.gov.pl/s.php?id=1273, Accessed 2009-07-27.

[69] Loco citato.

[70] L.c.

[71] L.c.

Considering the fact that this "team" has been established by he Polish government and taking into account that the issues that group is dealing with **are** highly political by its nature, Tusk's statement has to be called at least "surprising".

Bioethics, especially in regards of Regenerative Medicine in Poland is a topic as political as in any other EU-state – and even more. The political and influential role of the Roman Catholic Church in this regard cannot be overestimated in terms of influence on bioethical leading arguments and respective political decisions on national level. Therefore, it keeps an open question if a Polish National Ethics Commission can disregard Church interests without causing political struggles of large dimension.

Analyzing inter-alia the question why there is no National Ethics Commission in Poland, *Szawarski* describes Poland superbly this way: "Poland is predominantly a catholic country. For someone who has known "the moral truth" there is no need for discussion and moral compromise. It is known a priori that e.g. some reproductive activities are morally wrong and stem cell research should never be morally approved".[72]

Considering the importance of "Catholic positions" in Poland, the Tusk administration has made a smart tactical move in establishing the chairman of the above mentioned "team" with Jaroslaw Gowin. Gowin is member of the governing party **and** president of the *Tischner European University*, named after and dedicated to his friend and mentor, the late *Józef Tischner*, a famous liberal Catholic priest and significant philosopher, still very much venerated in Polish public.

Evaluated politically, the person of Gowin seems to represent in a unified way both, party positions and catholic interests. This way, in bioethics Gowin is a precious tactial personnel and provides valuable political leeway for the government, probably not been given in case of another s.

We have mentioned these facts on the "team for Bioethics convention" and the connected circumstance so exhaustingly for it shows a basic and general aspect of any official ethical work in Poland: the "church factor" has to be at least considered when official steps in bioethics are done. Especially in connection with Regenerative Medicine, in Poland statements (given or being missed) from politics and academia often can be characterized as "preemptive obedience" to church positions, better not to get in conflict with.[73]

All hopes for open discussions on ethics of Regenerative Medicine are pinned on enlightened representatives of Biomedical Science in Poland – although the political weight of Science – in European comparison – in Poland has to be called weak – yet.

---

[72] Szawarski (2004).

[73] Just within the last few years, we have observed that members of the Scientific community have been daring advocating ethical positions in opposition to church positions. Too, it has to be mentioned that recently provocations, also in bioethical sense, of the politician Mr. Palikot, have brought in the sequel a more open discussion e.g. regarding in-vitro-fertilisation and other also bioethical topics. A new term in Poland for unusual and provoking statements is named after him: "*palikotyzacja*", it keeps an open question whether "palikotyzacja" can help establishing an open discussion generally on ethical aspects of Regenerative Medicine – we do not wish to exclude this possibility here and now.

Nevertheless, first of all, in a European frame Polish biomedical Science has got quite a comparable quality level and Regenerative Medicine is an interesting topic for Polish Scientists. Secondly, Science in Poland enjoys traditionally the privilege of freedom and independence, and thirdly there are certain versatile hints that Polish Science insists more and more on its charted right of freedom of Science and its connected ethical responsibility coming along with that freedom.

The transition of bioethical debates to administrative and political levels and the establishment of a working National Bioethics commissions as units of counsel and check-ups – like in France or German – can hardly be expected within the decade. In Poland, Regenerative Medicine is a topic of Science and the political relevant public opinion on it keeps distance to connected goals and hopes of Regenerative Medicine. A reason to be mentioned for that is also the comparably low rate of available information to public on bioethics and Regenerative Medicine. Therefore, the political class will be extremely cautious dealing with this and other related topics, and an establishment of more or less powerful bioethical commissions like in France and Germany is as improbable as the development of respective liberal laws and liberal regulations for Regenerative Medicine.

45.2.3 The Situation of Ethics Commissions in Germany

In Germany in 2001 the Schröder administration has established the National Ethics Council (Nationaler Ethikrat), changed in 2008 to the German Ethics Council (Deutscher Ethikrat). This unit has been the German version of a National Bioethics Committee, with the tasks to give counsel to the parliament, to raise public discussions and in general to be a central forum for bioethics.[74]

Although the importance of this unit is beyond question, another national ethics committee is of more practical relevance.

"The Central Ethics Committee for Stem Cell Research (Zentrale Ethik-Kommission für Stammzellenforschung, henceforth ZES) was established in 2002 with the enactment of the Stem Cell Act. The 18 members and deputy-members of the ZES are appointed by the German FederalSIC! for 3-year periods. The ZES is charged witSIC! the task of reviewing and evaluating applications for import and use of human ES cells according to the Stem Cell Act and has to submit a written opinion on each application to the licensing authority, the RKI (Robert-Koch-Institute – *the author*).

The Office of the ZES organises the meetings of the ZES, supports the members and deputy members of the committee in the assessment of applications according to the Stem Cell Act and coordinates the collaboration between the ZES and the licensing authority.

[74] See also: http://www.ethikrat.org/de_der/ethikratgesetz.php

The Central Ethics Committee for Stem Cell Research submits an annual report to the Federal Ministry of Health."[75]

There are some open questions connected with the work, procedures of making decisions and structure of this commission, partially already mentioned above: the question whether an ethics commission with sovereign function shall be established by the government, the structure of the commission, disregarding important pressure groups, opinion makers and NGOs, and the question of involvement of representatives of Catholic Church, when it is known that it principally denies stoutly the idea of embryonic stem cell research.[76]

Nevertheless, based inter alia on the yearly reports[77] the work of ZES has to be evaluated an outstanding example of excellent work of a bioethics commission.

First of all, in this commission main relevant specialists for decisions making processes on an ethical level based on existing law come together.[78]

The possible risk that the scientific horizon and possible future consequences of a planned research object keeps unclear or undiscovered is excluded. Scientific details of proposed projects can be explained by present scientists. This way the complete scientific setting can be considered in the work of the commission. The same is valid for the often complex and "delicate" ethical argumentation from philosophical and theological point of views.

Secondly, the idea that Practical Ethics has to come to conclusions is considered, for the goal of the commission's work is to evaluate concrete research projects.

Thirdly, this commission's decisions are finally based on existing law and a preferable bypass of ethics and law is guaranteed.[79,80]

---

[75] RKI (2009).

[76] The fact that in this commission the Protestant and Catholic Church are represented by Professors of Theology does de facto not speak against their designation as "church representatives. In Germany Professors of Theology need permission by church, the "missio canonica", respectively the "Vokation" for filling their academic positions. Therefore, it might make a political difference between direct involvement of the Christian churches and the involvement of academic Theologians, but not in the matter. Academic Theologians are obliged to represent the official church guidelines and principles.

[77] Loco citato.

[78] For any concrete impact of bioethics it is a fatal mistake if bioethics is evaluated a discipline of philosophy or theology! The competence of evaluating ethical dimensions of biomedical Science is given to scientists as well. Therefore, "ethics" is not an esoteric but exoteric discipline – not for specialist from Humanities only but –for all society, including scientists taking over responsibility by reflecting ethics of their own work! ZES is an excellent example how scientists "can be forced" dealing with ethics of their work; something that too rarely is still the case!

[79] As mentioned before: although a research project can fulfil all legal requirements, in spite of that, by a commission like ZES it can be evaluated as unethical. This possibility makes the essential difference between a purely legal and an ethics commission; this way it builds a much "finer instrument" and therefore an "appropriate instrument" dealing ethically with issues from Biomedicine.

[80] In an edition published on the occasion of the fifth anniversary Siep (2008a) deals critically with the German Stem Cell law and "The view of the German Central Ethics Committee for Stem Cell Research" (l.c.). He states that "according to the German Stem Cell Act the Central Ethics Committee for Stem Cell Research (ZES) advices the competent authority (Robert Koch Institute)

Interesting details about the concrete ethical debates within the commission keep classified: "For the proposal-discussion is part of an approval-procedure, the "daily routine" of the commission's work requires strict confidence."[81]

Therefore, exact information on the course of the ethical debate keeps unknown, as well as exact information on respective decision making processes within the group.

Till now, the Robert-Koch-Institute, as the hosting public body of the commission, has followed all evaluations of the commission:

"All the projects supported by ZES were approved by RKI."[82]

Too, there is not even one case known, in which an objection against the commission's evaluation has followed from the side of the applicant.

A fact that surprises in this context is the relatively low total number of proposals the commission has dealt with.

"In its work now spanning 6 years, ZES has deliberated on a total of *38 applications* for the import and/or use of human embryonic stem cells and *three applications for extensions* to already approved projects. In total *41 opinions* were handed down, *39 of them were positive*."[83]

After longstanding national discussion about the absolute necessity of large-dimensioned high-end research in the field of embryonic Stem Cell research (promoted not least by strong voices from Science), it could have been expected that ZES would have faced a much higher number of proposals to deal with.

There are several possibilities for the low number of applications:

- There are not enough stem cell researchers in Germany for large-dimensioned Stem Cell Research
- Large-dimensioned Stem Cell Research was a political figure of speech, designed for a political struggle on more than Stem Cell Research, but on the fight against any restrictions in Science at all
- The legal regulations, based on nation-wide ethical discussions are too restrictive

If the last point might be the right answer, it could be concluded that ethical restrictions put into law, expressed in the German Stem Cell Act, has lead to a situation in which German Science cannot develop due to its full potency by ethical "handcuffs".

---

as to whether an application to import human embryonic stem-cells for research is "ethically justifiable" ("ethisch vertretbar"). The law does indeed specify some conditions of this justification, but without precisely defining them." (l.c.). Generally, the question of "broad" legal formulation and the possible leeway in interpretation from ethical side is quite a difficult one that requires another publication than the present one; however, from a legal point of view the question is "easy" to answer, regarding connected other laws and the spirit of basic rights formulated in the German Constitution (Grundgesetz).

[81] Siep (2008b).

[82] ZES (2008).

[83] ZES (2008).

Here and now, we keep this point open for further discussions, as well as the question if less- or un-restricted Science in the field of embryonic Stem Cells can be desirable at all.

45.2.4 The Situation of Ethics Commissions in France

In France the landscape of ethics commission is heterogeneous.

There are several commissions with different tasks, but the one of highest importance is the COMITÉ CONSULTATIF NATIONAL D'ÉTHIQUE POUR LES SCIENCES DE LA VIE ET DE LA SANTÉ (henceforth CCNE).

Also due to the restrictive law situation, a direct work of the CCNE with ethical aspects of concrete research projects from within the field of Regenerative Medicine is not given.

The CCNE describes its work with the following statement ending with a remarkable, although cryptic, statement:

"Casting light on the progress of science, raising new societal issues and looking at change from the perspective of ethics: such is the mission of the French National Consultative Ethics Committee on Health and Life Sciences – at the very heart of society's present-day debates. The CCNE always encourages thinking on bioethics by contributing to the debates – never by stealing them."[84]

The French government is at liberty to request or not bioethical counsel at the CCNE. Additionally, public research institutions can request counsel in bioethical questions. The CCNE gives statements on relevant ethical questions from Biomedicine, including Regenerative Medicine, e.g. "N°052 Opinion on the creation of human embryonic organ and tissue collections and their use for scientific purposes (1997-03-11)".[85]

Sicard[86] intimates that the statements of CCNE keep without deeper impact on the political level and *Deutsch*[87] claims explicitly that the influence of CCNE on the national legislation has kept restricted only.

One reason for that might be seen in the fact that in France political issues, and bioethics is definitely such, are treated finally as such by the political class of the executive. In an online interview *Sintomer* pleads in favour of more "Participatory democracy" in France based on his analysis that in the presidential system "(…)in practice there is hardly any counterweight to the omnipotence of the executive."[88]

The importance of the role of ethics consultants, politicians and commissions in France and Germany is still matter of discussions; in Germany there were big political argues in connection with the National Ethics committee, e.g. by the former

[84] CCNE (2009a).

[85] CCNE (2009b).

[86] Sicard (2007).

[87] Deutsch (2007).

[88] Sintomer (2009).

chairman of the committee of inquiry "Ethics and Law of Modern Medicine" of the German Bundestag, Mr. Röspel. Mr. Röspel criticizes inter alia the low influence of members of parliament in a the National Ethics council, the reformed National Ethics commission.[89] This argue shows quite a dilemma connected with National Ethics committees and commissions: The legitimation of National ethics unit. On the one hand the idea that ethics specialists and specialists of Biomedicine shall be members is obvious; on the other hand the commission members have no democratic legitimation at all, and in no case they are representatives speaking for the people, in what way ever. Representatives of the people are solely members of the parliaments. In the French presidential system the meaning of the parliament may be smaller than in Germany and the meaning of the executive comparably greater than in Germany[90] – however, the point is that they all are elements established finally by democratic processes, meanwhile a national ethics committee established by who ever is not.

The French CCNE has got remarkable aspects concerning its structure. First of all, not only academic experts of Ethics, Theology and Science are members of the commission. The term "expert" is meant in a broader sense. *Sicard* explains: "The term "expert" does not fit completely to French circumstances. Whereas different members can be considered as experts for their subject, especially the ones coming from research, one has to point out that 19 members have not been chosen only for their expertise but for their interest in ethical questions.(…)

On the other hand, the membership of five personalities representing big philosophical and religious directions in France makes a pluralistic representation of different ideologies possible."[91]

Deutsch reports: "It (the CCNE-the author) consists of 37 members, among them 15 researchers, further a Catholic, a Protestant, a Jew, a Muslim and a Marxist."[92]

The CCNE considers the idea of pluralism in a National Ethics Committee and hint on structural questions of National Ethics Committees. First of all, in bioethics committees there is an often existent concentration on academic specialists that is generally debatable considering the societal dimensions of ethical questions * e.g. in connection with Regenerative Medicine.

Secondly, all Western States of the EU, including Germany, have a relative high number of Muslims in their population that shall be represented in National Ethics Committees.

Thirdly, the cultural roots of Europe are Judaeo-Christian, although the Jewish population is currently comparably low in Europe; therefore, a participation of Jewish representatives in National Ethics Committee may be appropriate.

Fourthly, the legitimation of a National Ethics commission is – as mentioned – a difficult issue; nevertheless, under aspects of the pluralistic self-image of European

[89] Deutschlandradio (2006).

[90] See also Sentimer (2009).

[91] Sicard (2007).

[92] Deutsch (2007).

democracies, an embedment of other influential ideologies and in certain cases of minorities (disabled, gays etc.) shall be indicated.

It also can be called appropriate that gender related bioethical issues make it necessary to consider gender-representatives as members of a National Ethics Committee.

Furthermore, the possible role of NGOs in settings of National Ethics Commission is still to be discussed.

If a National Ethics Committee shall mirror a pluralistic society, more variations in its setting is indicated! The French CCNE teaches an at least more pluralistic model of composition of a National Ethics commission.

45.2.5 Conclusions

The character of National Bioethics Committees, Commissions and Councils in France, Germany and Poland is directly not comparable.

In France the influence of the National ethics commission on political decisions is comparably low, in Germany in comparison probably most influential and in Poland the question of the importance and duties of a possible National bioethics unit not decided yet.

We have found many points for critics in the different national structures of bioethics commissions, and we recommend continuing the discussion on the structures, on the democratic legitimation and on the intransparant decision making processes of ethics commissions.

However, we have come to the conclusion that the German ZES is currently the most advanced model of dealing **conclusion-oriented** with ethical questions of Regenerative Medicine based on law.

References

Agence de la Biomédecine (Biomedicine Agency) (2008) Application Report – Bioethics Law of August 6, 2004, Report to the Minister of Health, Youth, Sports and Associative Life, October 2008, pp 58–80. http://www.agence-biomedecine.fr/uploads/document/rapport-bilan-LB-oct2008.pdf. Accessed 12 Sept 2009

Bundesministerium für Gesundheit (2002) Verordnung über die Zentrale Ethik-Kommission für Stammzellenforschung und über die zuständige Behörde nach dem Stammzellgesetz. Bundesgesetzblatt 2002 Teil 1 Nr. 49 23. Juli 2002. http://217.160.60.235/BGBL/bgbl1f/bgbl102s2663.pdf. Accessed 7 July 2009

CCNE (2008) COMITÉ CONSULTATIF NATIONAL D'ÉTHIQUE POUR LES SCIENCES DE LA VIE ET DE LA SANTÉ, Opinion no.105 "Questions for the Estates General on Bioethics". http://www.ccne-ethique.fr/docs/avis105anglais.pdf. Accessed 12 Sept 2009

CCNE (2009a) COMITÉ CONSULTATIF NATIONAL D'ÉTHIQUE POUR LES SCIENCES DE LA VIE ET DE LA SANTÉ. http://www.ccne-ethique.fr/theccne.php. Accessed 29 July 2009

CCNE (2009b) COMITÉ CONSULTATIF NATIONAL D'ÉTHIQUE POUR LES SCIENCES DE LA VIE ET DE LA SANTÉ. http://www.ccne-ethique.fr/inc_recherche.php?langue=2. Accessed 29 July 2009

Code de la santé publique (Textes – Commentaires – Jurisprudence – Conseils pratiques – Bibliographie) (2009) sous la direction de F. DREIFUSS-NETTER, Litec, coll. Juriscode. Juriscode, éd. 2009

CONSEIL D'ETAT (Council of State) (1988) De l'éthique au droit, La Documentation Française, Notes et études documentaires

CONSEIL D'ETAT (Council of State) (2008) "The revision of bioethical laws", Study adopted by the General Plenary Session, Les études du Conseil d'Etat, 2008. http://www.conseil-etat.fr/cde/media/document//etude-bioethique_ok.pdf. Accessed 12 Sept 2009

Conseil d'Orientation de l'Agence de la Biomédecine (Orientation Council of the Biomedicine Agency), Contribution of the Agence de la Biomédecine Orientation Council pertaining to the bioethics law revision preparatory debates. Lessons of experience (2005–2008) and Questionings. http://www.agence-biomedecine.fr/uploads/document/bilanLB-partie3.pdf. Accessed 12 Sept 2009

Deutsch E (2007) Medizinrecht, Berlin. http://books.google.de/books?id=PCePIh9xg_cC&pg=PA629&lpg=PA629&dq=ethikkommission+frankreich&source=bl&ots=e5d9AL8SPC&sig=zu_7PLzpIk7yz7GQK-Zs9qS2-NM&hl=de&ei=gVJwStWiOMHDsgbEvaWcBQ&sa=X&oi=book_result&ct=result&resnum=4. Accessed 29 July 2007

Deutscher Ethikrat (2004) Opinion "Biobanks for research". http://www.ethikrat.net/_english/publications/Opinion_Biobanks-for-research.pdf. Accessed 12 Sept 2009

Deutscher Ethikrat (2007) Opinion "Should the Stem Cell Law be amended?". http://www.ethikrat.org/_english/publications/Opinion_Should_the_Stem_Cell_Law_be_amended.pdf. Accessed 12 Sept 2009

Deutschlandradio (2006) Deutscher Ethikrat in der Kritik. Interview with René Röspel. http://www.dradio.de/dkultur/sendungen/thema/516132/. Accessed 29 July 2009

Etats Généraux de la Bioéthique (General Estates on Bioethics) (2009) Final report drafted by Graf A, 1st July 2009. http://www.etatsgenerauxdelabioethique.fr/uploads/rapport_final.pdf. Accessed 12 Sept 2009

Fagniez P-L (2006) Cellules souches et choix éthiques – Rapport au Premier Ministre, La Documentation Française, Collection des rapports officiels

Fischer F (2000) Citizens and experts in biotechnological policy. The consensus conference as alternative model. In: Barben D (ed) Biotechnologie – Globalisierung – Demokratie. Politische Gestaltung transnationaler Technologieentwicklung, Berlin

Fuchs M (2001) Ethikräte im internationalen Vergleich. Modelle für Deutschland? Arbeitspapier herausgegeben von der Konrad-Adenauer-Stiftung 12/2001, St. Augustin

Haßmann H (2002) Embryonenschutz im Spannungsfeld internationaler Menschenrechte, staatlicher Grundrechte und nationaler Regelungsmodelle zur Embryonenforschung, Berlin

Joss S (ed) (1995) Public participation in science. The role of consensus conferences in Europe. Science Museum, London

Joss S (1999) Introduction: public participation in science and technology policy- and decision-making: ephemeral phenomenon or lasting change. Sci Pub Pol 26(5):290–293

Keller C (2009) Embryonal oder adult. http://www.gen-ethisches-netzwerk.de/gid176_hauskeller. Accessed 10 July 2009

Koch C (1990) Ethik-Kommissionen – Ein Ersatz. In: Füllgraf G et al (ed) Wissenschaft in der Verantwortung: Möglichkeiten der institutionellen Steuerung, Frankfurt am Main

Krippner B (2004) Bioethik-Kommissionen in Deutschland Bioethik-Kommissionen in Deutschland – Ein Überblick. In: MRM —MenschenRechtsMagazin Heft 3/2004, pp 239–260

Nocke J (1995): Organisierte Moral. Das Beispiel der sogenannten Ethikkommissionen. In: Nahamovitz et al (ed) Politik – Verfassung – Gesellschaft: Traditionslinien und Entwicklungsperspektiven, Baden-Baden

Office Parlementaire d'Evaluation des Choix Scientifiques et Technologiques (Parliamentary Office for Scientific and Technological Options) (2006) Report on research into the functioning of human cells. Report no. 3498 (Assemblée Nationale), drafted by M. Alain CLAEYS. http://www.assemblee-nationale.fr/12/rap-off/i3498.asp. Accessed

Office Parlementaire d'Evaluation des Choix Scientifiques et Technologiques (Parliamentary Office for Scientific and Technological Options) (2008–2009) Report on the evaluation of the

appliance of the bioethics law of August 6, 2004. Report no. 1325 (Assemblée Nationale) and no. 107 (Sénat), drafted by M. Alain CLAEYS and M. Jean-Sébastien VIALATTE. http://www.senat.fr/rap/r08-107-1/r08-107-11.pdf. Accessed 6 Aug 2004

Pichlmaier H et al. (2003) Die Zentrale Ethik-Kommission bei der Bundesärztekammer. Wiesing

Rippe K (ed) (1999) Angewandte Ethik in der pluralistischen Gesellschaft, Freiburg

Schlenke T et al (2008) The impact of the German Tissue Act on the manufacturing of autologous and allogeneic stem cell preparations. Transfus Med Hermother 35:446–452

Schröder M (2001) Die Institutionalisierung des Nationalen Ethikrates: Ein bedenklicher Regierungsakt? Neue Juristische Wochenschrift 30:2144–2146

Sicard D (2007) Fragebogen zu Ethikkomissionen. http://webarchiv.bundestag.de/archive/2007/0108/parlament/gremien/kommissionen/archiv14/medi/medi_di_stell/Sicard_dt.pdf. Accessed 28 July 2007

Siep L (2008a) In diesem Sinne ethisch vertretbar: Zum Ethikverständnis der Zentralen Ethik-Kommission für Stammzellenforschung. Bundesgesundheitsblatt – Gesundheitsforschung – Gesundheitsschutz 51(9):950–953. http://www.springerlink.com/content/6tk6u03358572221/. Accessed 29 July 2009

Siep L (2008b) In diesem Sinne ethisch vertretbar: Zum Ethikverständnis der Zentralen Ethik-Kommission für Stammzellenforschung In: Bundesgesundheitsblatt – Gesundheitsforschung – Gesundheitsschutz 51(9):947–949. http://www.springerlink.com/content/1un1g3q62h633142/?p=9423b95c984c41c5b6f69c4520bc63ec&pi=0. Accessed 29 July 2009

Sintomer Y (2009) Democracy is a system constantly called into question. http://www.diplomatie.gouv.fr/en/france_159/label-france_2554/label-france-issues_2555/label-france-no.-66_5559/feature.-france-politics-in-action_5589/background-and-election-rules_5590/democracy-is-system-constantly-called-into-question_8979.html. Accessed 28 July 2009

Starck L (2006) Embryonic stem cell research according to German and European law, 41 EUROPARECHT 1. http://www.germanlawjournal.com/pdf/Vol07No07/PDF_Vol_07_No_07_625-656_Articles_Starck.pdf

Szawarski Z (2004) Research ethics in Poland. Theory and practice. Presentation at good scientific practice – BSRUN workshop on research ethics, Turku, Finland, 12–13 November 2004. http://bsrun.utu.fi/tiedostot/Turku_Ethics_2004/Szawardski%20in%20Poland.ppt. Accessed 27 July 2009

Taupitz J (2003) Ethikkommissionen in der Politik: Bleibt die Ethik auf der Strecke? Juristenzeitung 58(17):815–821

Taupitz J (2004) Germany: current legislation. In: Biomedical Research, Council of Europe publ

Thiele F (2001) Bio-policy and the place of institutionalized ethics in bioethical decision-Making. Europäische Akademie zur Erforschung von Folgen wissenschaftlich-technischer Einrichtungen (ed) Newsletter, Akademie-Brief 2/2001

Toellner R (ed) (1990) Die Ethik-Kommission in der Medizin: Problemgeschichte, Aufgabenstellung, Arbeitsweise, Rechtsstellung und Organisationsformen medizinischer Ethik-Kommissionen, Stuttgart

Van den Daele W (1990) Regeldurchsetzung und Normbildung bei der Kontrolle biomedizinischer Forschung. Zur Funktion von Ethikkommissionen in der Bundesrepublik Deutschland. Kölner Zeitschrift für Soziologie und Sozialpsychologie 42

Von Blumenthal J (2003) Auswanderung aus den Verfassungsinstitutionen. Kommissionen und Konsensrunden. Aus Politik und Zeitgeschichte B 43:9–15

Vowe G (1989) Die Sicherung öffentlicher Akzeptanz. Verlauf, Struktur und Funktion der Enquête-Kommission "Chancen und Risiken der Gentechnologie". Politische Bildung 22(2):49–62

Wiesing U (ed) (2003) Die Ethik-Kommissionen – Neuere Entwicklungen und Richtlinien, Köln

World Medical Association (2008) Declaration of Helsinki. http://www.wma.net/e/policy/pdf/17c.pdf. Accessed 22 July 2009

Zentrale Ethikkommission bei der Bundesärztekammer (1994) Statut der Zentralen Kommission zur Wahrung ethischer Grundsätze in der Medizin und ihren Grenzgebieten. http://www.zentrale-ethikkommission.de/page.asp?his=0.2.29&all=true. Accessed 7 July 2009

Zentrale Ethikkommission für Stammzellenforschung (2003) Tätigkeitsbericht der Zentralen Ethik-Kommission für Stammzellenforschung (ZES)Sechster Bericht nach Inkrafttreten des Stammzellgesetzes (StZG) für den Zeitraum vom 01.12.2007 bis 30.11.2008. http://www.bmg.bund.de/cln_100/nn_1168248/SharedDocs/Downloads/DE/Standardartikel/S/Glossar-Stammzellforschung/Glossarbegriff-Stammzellforschung__Sechster-T_C3_A4tigkeitsbericht,templateId=raw,property=publicationFile.pdf/Glossarbegriff-Stammzellforschung_Sechster-Tätigkeitsbericht.pdf. Accessed 7 July 2009

ZES (2008) Sixth report after enactment of the Stem Cell Act (StZG) http://www.rki.de/cln_153/nn_207098/EN/Content/Institute/DepartmentsUnits/StemCell/6th-ReportZES,templateId=raw,property=publicationFile.pdf/6th-ReportZES.pdf. Accessed 29 July 2007

Zoll A (2005) Can we talk about a legal statute on stem cells? Gazeta Lekarska, No. 2005-02, Debata. http://www.gazetalekarska.pl/xml/nil/gazeta/numery/n2005/n200502/n20050221

Index

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8, © Springer Science+Business Media Dordrecht 2013